Effects Of Cyclosporin A On Peripheral Immune Tolerance

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ABSTRACT

Cyclosporin A (CsA) is a potent immunosuppressive agent that can alter immunological tolerance in deleterious ways by provoking autoimmunity, or in beneficial ways, by inducing permanent allograft tolerance. The mechanism(s) behind these paradoxical manifestations remain largely unknown. The lack of data on the effects of CsA on peripheral T cell anergy and deletion, two types of peripheral tolerance mechanisms, prompted this study. In mice treated with superantigens, we found that low doses of CsA (CsA^{lo}) blocked anergy induction, and that high doses (CsA^{hi}) enhanced the peripheral deletion. CsA^{hi} also enhanced the deletion produced by *in vivo* administration of anti-T cell-receptor (TcR) monoclonal antibodies, and delayed re-expression of the downmodulated TcR in the residual T cells. Based on these findings, it is conceivable that CsA enhances tolerance by promoting peripheral deletion, and conversely, induces autoimmunity by inhibiting anergy. The usage of CsA for the manipulation of immunological tolerance may have significant applications in clinical medicine.

ABRÉGÉ

La cyclosporine A (CsA) est un puissant immunosuppresseur qui affecte la tolérance immunitaire de diverses façons. Elle peut être nuisible, en provoquant certaines maladies autoimmunitaires, ou bien être bénéfique en induisant une tolérance permanente d'allogreffes. Les mécanismes sous-jacents à ces phénomènes paradoxaux sont encore peu connus. Le manque d'information quant aux effets de la CsA sur l'anergie et la délétion périphérique, deux formes de tolérance périphérique, ont motivé cette étude. Dans les souris traitées aux superantigènes, nous avons observé que de faibles doses de CsA (CsA^{faible}) bloquaient le développement de l'anergie, et que les fortes doses (CsA^{forte}) potentialisaient la délétion périphérique. Non seulement les CsA^{forte} augmentèrent la délétion retrouvée dans le traitement *in vivo* aux anticorps monoclonaux contre le récepteur cellulaire T (RcT), mais elles ont également retardé la réexpression des RcT internalisés chez les cellules T résiduelles. En se basant sur ces résultats, il est probable que la CsA promeut la tolérance par la potentialisation de la délétion périphérique, et qu'elle induit le développement de l'autoimmunité en inhibant l'anergie. L'utilisation de la CsA dans le but de manipuler la tolérance immunitaire peut avoir d'importantes applications en médecine clinique.

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ABBREVIATIONS

A

Ag	-Antigen
APC	-Antigen Presenting Cell

C

c.p.m.	-Counts per minute
CsA	-Cyclosporin A
Ctl	-Control

D

DP	-Double Positive
DPA	-Diphenylamine
DTH	-Delayed-Type Hypersensitivity

F

FITC	-Fluorescein Isothiocyanate
FK	-FK-520 (L-683,590)
FKBP	-FK-506-Binding Protein

G

GVHD	-Graft-Versus-Host-Disease
GVHR	-Graft-Versus-Host-Reaction

I

IL	-Interleukin
IFN	-Interferon
i.p.	-Intraperitoneal
i.v.	-Intravenous

L

LCMV	-Lymphocytic Choriomeningitis Virus
LN	-Lymph Node

M

MHC	-Major Histocompatibility Complex
MLC	-Mixed Lymphocyte Culture
Mls	-Minor Lymphocyte Stimulating Genes/Proteins

M

MMTV-7	-Mouse Mammary Tumor Virus
MoAb	-Monoclonal Antibody

N

NF-AT	-Nuclear Factor of Activated T cell

O

OD	-Optical Density
OO	-Olive Oil

P

PE	-Phycoerythrin
PKC	-Protein Kinase C
PMA	-Phorbol Myristate Acetate

R

RAPA	-Rapamycin
RBC	-Red Blood Cell

S

SEA	-Staphylococcal Enterotoxin A
SEB	-Staphylococcal Enterotoxin B
SN	-Supernatant
SuperAg	-Superantigen

T

TcR	-T cell Receptor
T_H	-T helper
TNF	-Tumor Necrosis Factor
Tx	-Thymectomized

W

WF	-Wistar Furth

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To my mother,

A ma mère,

Laure Bélair-Vanier

CHAPTER I.

GENERAL INTRODUCTION

1. T cell Tolerance

The immune system has evolved to seek out and destroy invading pathogens. In the process, the immune system has to discriminate 'self' and 'non-self'. Failure to recognize self molecule is essential to avoid the damaging consequences of autoimmunity. This knowledge is not encoded in the germline, and therefore, the T cell has to be 'educated'. A good part of this education takes place during the T cell's development. Thus, the thymus, site of the T cell maturation, is the first line of protection. It is there that the thymocytes bearing receptors that recognize self antigens can be physically or functionally eliminated (Kappler, J.W., Roehm, N., and Marrack, P., 1987; Kisielow, P., Blüthmann, H., Staerz, U.D., and von Boehmer, H. 1988a). Since it appears unlikely that all self molecules would be expressed in the thymus, some extrathymic mechanisms (termed peripheral) are required for maintaining self tolerance. Based on our current understanding, peripheral tolerance can be achieved in three different ways (Miller, J.F.A.P., and Morahan, G., 1992). First, deletion, where mature T cells, which exhibit high affinity to self antigens, are eliminated. Second, anergy, accomplished by functional silencing of reactive lymphocytes due to inadequate or missing costimulatory signal(s) when self molecules get involved. Third, functional inhibition of self reactive T cells by other cells (suppression/immunoregulation). All the mechanisms of T cell tolerance discussed in this chapter are summarized

in table 1.

Before discussing recent progress, we will briefly review some of the historical developments that have led up to our present understanding of tolerance.

Table 1. **Mechanisms of T cell tolerance**

LOCATION	TYPE	DESCRIPTION	CONTRIBUTION TO SELF TOLERANCE	PAGE
INTRATHYMIC	Positive selection	Thymocytes selected upon proper recognition of MHC molecules	Avoids anarchic targeting by T cell	10
	Negative selection	Thymocytes with excessive avidity for MHC molecules are eliminated	Prevents the formation of autoreactive T cells	12
EXTRATHYMIC (PERIPHERAL)	Anergy	Inadequate T cell activation results in functional silencing of mature T cells	Limits T cell activation to antigen presented by immunocompetent antigen-presenting-cells	15
	Deletion	Overreactive T cells or autoreactive T cells are physically eliminated	Prevents uncontrolled or unwanted T cell responses	18
	Immunoregulation	Active suppression of T cell responses by secreted factors	Controls T cells responses and/or orients T cells to either cellular or humoral response	19

Early History of Tolerance

The first concept of immune tolerance takes its origin back to the early 20thcentury. At a time when immunologists were injecting all substances that came to hand to experimental animals for the purpose of raising specific antibodies, some found that no antibodies could be produced against autologous components. It was then that Paul Erhlich formulated his famous dictum of *horror autotoxicus*, implying the need for a 'regulating contrivance' to prevent autoantibodies production (Erhlich, P., and Mogenroth, J., 1910).

We had to await Traub's discovery (Traub, E., 1938) to obtain the first demonstration of specific tolerance to endogenous antigens. By inoculating mice *in utero* with lymphocytic choriomeningitis virus (LCMV), he found that high titers of virus persisted in blood and tissues throughout life, and despite this, no antibody to this virus was made. But the word tolerance came to use with Owen (Owen, R.D., 1945), with his 'experiment of nature', where dizygotic twin cattle exchanged hemopoietic cells via anastomosed placental blood vessels. Each offspring was chimeric, containing erythrocytes of both its genotype and that of its twin. This was surprising, considering that all animals make strong immune response when injected with blood cells from other members of the species. Comparable experiments were later performed with other species with the same

conclusion (Hašek, M., and Hraba, T., 1955; Mintz, B., and Silvers, W.K. 1967).

After Owen's demonstration of mosaic twins, Burnet and Fenner (Burnet, F.M., and Fenner, F., 1949) predicted that an antigen introduced into the body during embryonic life, before the immune system had developed, would trick the system and would always be mistaken for self, even if reintroduced later in adult life. Burnet and colleagues failed to produce experimental evidence in favor of their postulate (Burnet, F.M., Stone, J.D., and Edney, M., 1950), but their prediction soon found support in the classical experiments of Billingham and colleagues (Billingham, R.E., Brent, L., and Medawar, P.B., 1953). They induced immunological tolerance to skin allografts by neonatal injection of allogeneic cells in mice. These results were in accordance with the clonal selection theories of Burnet (Burnet, F.M., 1957) and Talmage (Talmage, D.W., 1957). If immunity is a selective activation of certain clones in response to a given antigen, then tolerance could be obtained by deletion of self reactive clones. So antigens encountered after birth would activate specific clones to proliferate, whereas antigen encountered before birth would result in clonal deletion of these 'forbidden clones'. This idea of repertoire purging was further refined by Lederberg (Lederberg, J., 1959) who suggested that immature lymphocytes were subject to 'clonal abortion' upon antigen recognition, whereas mature cells would be activated. Strong evidence was obtained in support of this notion, which was further developed to include clonal anergy, the nondeletional inactivation of

reactive cells (Nossal, G.J.V., 1983).

Subsequent experiments demonstrated that adult animals too could be rendered nonresponsive to foreign antigens. For example, lethal irradiation and bone marrow transplantation showed that the repopulation of the immune system was an occasion for tolerance induction, and was seen as a recapitulation of ontogeny (Loutit, J.F., 1959). Eventually, tolerance theories came to encompass the concept that indefinite maintenance of tolerance had to depend on continuing immunoregulatory event(s) to account for the continuing stream of new immunocytes maturing throughout life in the primary organs (Nossal, G.J.V., 1974). However, it became difficult to interpret most of the other experiments of the time involving adult induction of tolerance with the existing theories. Induction of a nonresponsive state could be induced by large or very low doses of antigen (Mitchison, N.A., 1964); administration intravenously or orally rather than subcutaneously or intramuscularly (Chase, M.W., 1946; Battisto, J.R., Bloom, B.R., 1966); deaggregated rather than aggregated forms of the antigen (Dresser, D.W., 1962); impairment of the immune system with anti-lymphocyte serum, or drugs, such as cyclophosphamide, at the time of antigen administration (reviewed in Klein, J., 1982). It gradually became clear that a better knowledge of immunological reactions was required for understanding the cellular mechanisms involved in tolerogenesis. With the discoveries of the crucial role of the thymus (Miller, J.F.A.P., 1961) and the bursa of Fabricius (Szinberg, A., and Warner,

N.L., 1962), and the revelation of T cell-B cell cooperation in antibody production (Claman, H.N., Chaperon, E.A., and Triplett. R.F., 1966; Miller, J.F.A.P., and Mitchell, G.F., 1967; Rajewsky, K., Schirrmacher, V., Nase, S., and Jerne, N.K., 1969), all tolerance phenomena had to be reexamined from the viewpoint of whether the unresponsiveness was induced in T cells, B cells or both.

A further discovery revolutionized our conception of tolerance mechanisms. It was found that major histocompatibility complex (MHC) gene products acted as restriction elements in T cell-mediated responses (Rosenthal, A.S., and Shevach, E.M., 1973; Zinkernagel, R.M., and Doherty, P.C., 1974; Shearer, G.M., 1974). It was subsequently shown that T cells recognize relatively short peptide fragments which become wedged in the groove of the MHC molecules on the cell surface of antigen presenting cells (APC) (Bjorkman, P.J., Saper, M.A., Samaroui, B. Bennett, W,S., *et al.*, 1987). The $CD4^+$ T cells (generally helper T cells) recognize peptides in class II MHC molecules, whereas the $CD8^+$ T cells (generally cytotoxic T cells) perceive peptide in association with class I MHC molecules (reviewed in Braciale, T.J., and Braciale, V., 1991). Since antigens had to be processed by APC and presented in association with MHC molecules, this implies that foreign antigens must compete with self molecules to obtain a successful display for T cell recognition. This brings the question as to how T cells get 'educated' to react against foreign peptides to eliminate invading pathogens and to not respond to peptides derived from self components.

Intrathymic Tolerance

The fact that neonatally thymectomized mice bearing foreign thymus grafts were specifically tolerant of thymus-donor type skin, but not of third party skin (Miller, J.F.A.P., 1962), was one of the first demonstrations suggesting that the thymus was responsible for the elimination of Burnet's forbidden clones. A better understanding of how the thymus was performing education and development of young T cells had to await the recent breakthroughs in T lymphocyte cloning (reviewed in Fathman, C.G., Fitch, F.W., Denis, K.A., and Witte, O.N., 1989), transgenic technologies (reviewed in Miller, J.F.A.P., 1989), and embryonic homologous recombination, more commonly referred to as gene knock-out (reviewed in Viville, S., 1992). The development of several techniques for obtaining large numbers of cells, all bearing a single antigen-specific T cell receptor (TcR), greatly facilitated the raising of monoclonal antibodies (MoAb) against these receptors (Allison, J., McIntyre, B., and Bloch, D., 1982; Haskins, K., *et al.*, 1983; Samelson, L., Germain, R., and Schwartz, R.H., 1983). The transgenic technologies have provided radically new approaches to investigate thymic events. Since defined genes, coding either for new antigens or for anti-self TcR, can be introduced into the genome, the immune system can be manipulated from within. Finally, instead of adding genes in the genome, like transgenic

technologies do, gene knock-out assesses the role of key molecules by stopping their synthesis. This allows us to ascertain the nature of the contribution of these proteins to T cell development by comparing knock-out animals to normal ones. With such tools at hand, we were now able to track T cells, modified or not by genetic technologies, and follow their fate within the thymus.

The vast majority of thymocytes die during their journey through the thymus (McPhee, D., Pye, J., and Shortman, K., 1979). Before $CD4^-CD8^-$ precursors commit themselves to mature $CD4^+$ or $CD8^+$ T cells, through $CD4^+CD8^+$ intermediates, they are subjected to two selective steps that shape the T cell repertoire: positive selection, which establishes restriction specificity for self-MHC molecules, and negative selection, which establishes tolerance to self-antigen by clonal deletion. Thymocytes that do not get selected by both selection processes are condemned to programmed cell death (apoptosis).

T cell recognition of antigen in association with self-MHC is not an inherent property of the developing T cells, but is 'learned' by positive selection. Studies on chimeric mice showed that it was the radioresistant thymic epithelium that imprinted MHC restriction on developing thymocytes (Bevan, M.J., 1977; Zinkernagel, R.M., Callahan, A., Althage, A., *et al.*, 1978). The requirement for positive selection in T cell ontogeny was directly demonstrated by gene knock-outs of class I and class II MHC molecules. It was found that mice lacking MHC class I had no mature $CD8^+$ in their lymphoid organs (Zijlstra, M., Bix, M., Simister,

N.E., Loring, J.M., *et al.*, 1990; Koller, B.H., Marrack, P., Kappler, J.W., and Smithies, O., 1990). The same observation was made in MHC class II deficient mice with respect to mature $CD4^+$ T cells (Cosgrove, D., Gray, D., Dierich, A., Kaufman, J.K., *et al.*, 1991). These experiments confirmed previous results obtained by MoAb injections of anti-TcR, anti-MHC or anti-CD4 into normal mice from birth (Kruisbeek, A.M., Mond, J.J., Fowlkes, B.J., *et al.*, 1985; Marrack, P, Kushnir, E., Born, W. *et al.*, 1988; Marušić-Galešić, S., Longo, D.L., and Kruisbeek, A.M., 1989; Fowlkes, B.J., Schwartz, R.H., and Pardoll, D.M., 1988). Positive selection was also demonstrated in TcR transgenic mice (Kisielow, P., Teh, H., Blüthmann, H., and von Boehmer, H., 1988b; Sha, W.C., Nelson, C.A., Newberry, R.D., *et al.*, 1988). From these experiments, it was concluded that positive selection is determined by a degree of binding avidity of the TcR to self-MHC molecules encountered on thymic epithelial cells. Double positive $CD4^+CD8^+$ (DP) thymocytes that successfully interact with self-MHC with their TcR are rescued from apoptosis and can pursue their developmental progression to mature lymphocytes. This selection process ensures that the mature $CD4^+$ T cells and mature $CD8^+$ T cells will recognize antigen when associated with self-MHC class II and class I molecules respectively. However, the positive selection allows the differentiation of T cells having high affinity TcR for self peptides presented in MHC molecules. The task of eliminating these potentially threatening thymocytes falls on the negative selection process.

The first insight supporting negative selection came from Kappler and colleagues (Kappler, J.W., Roehm, N., and Marrack, P., 1987). They observed that cells expressing a particular variable segment of the β chain of the TcR (Vβ17a), were a major subpopulation (9-14%) in mice that did not express a certain MHC class II molecule (namely I-E), and that this subpopulation was greatly reduced (<1%) in F_1 crosses with other mice that did express the I-E molecule. Low levels of Vβ17a$^+$ T cells were also observed in inbred strains expressing the I-E molecule. In addition, they made the critical finding that I-E$^+$ mice contain normal numbers of immature DP thymocytes expressing Vβ17a-TcR. Thus, the anti-I-E-thymocytes were deleted before they entered the mature single positive stage of their development. Identical observations were made in comparable systems (Kappler, J., Staerz, U., White, J., and Marrack, P., 1988; MacDonald, H.R., Schneider, R., Lees, R.K., *et al.*, 1988). The second system used to probe the negative selection events was the TcR transgenic mice. Kisielow and colleagues (Kisielow, P., Blüthmann, H., Staerz, U.D., and von Boehmer, H., 1988a) produced transgenic mice that contained an α and β TcR transgene, which together encoded a TcR specific for the male (H-Y) antigen presented by class I H-2D^d molecules. They observed that the thymi of males were atrophied, whereas female thymi were of normal size. The absolute number of CD4$^-$CD8$^-$ precursors expressing the transgenic receptor was the same in both male and female mice, showing that these cells were not the target of the deletion process.

However, they found that, at a later developmental stage, the DP thymocytes were virtually absent in the male. These conclusions reinforced the experiments performed in normal mice that showed that negative selection can be achieved before, or at, the DP stage. Similar observations were obtained in other transgenic systems (Sha, W.C., Nelson, C.A., Newberry, R.D., *et al.*, 1988; Pircher, H., Bürki, K., Lang, L., Hengartner, H., Zinkernagel, R.M., 1989).

The intrathymic sites and cells responsible for the selection processes were studied in recent works with transgenic mice. By engineering selective expression of I-E class II MHC molecules on different thymic stromal cells, investigators found that cortical epithelial cells positively selected thymocytes, whereas medullary macrophages, dendritic cells, and epithelial cells were responsible for the negative selection (Berg, J.L., and Davis M.M., 1989; Benoist, C., and Mathis, D., 1989). However, it would appear that clonal deletion is less effective with medullary epithelial cells (Blackman, M., Kappler, J.W., and Marrack, P., 1990; Hengartner, H., and Zinkernagel, R.M., 1992; Carlow, D.A., Teh, S.J., and Teh, H.S., 1992). Furthermore, medullary epithelium, but not cortical epithelium, may induce some T cells into an anergic state, as shown in two transgenic mouse lines. (Hoffmann, M.W., Allison, J., and Miller, J.F.A.P., 1992; Schönrich, G., Momburg, F., Hämmerling, G.J., and Arnold, B., 1992). It should be noted that many dissenting opinions remain as to how and when the

selection events occur (reviewed in Lo., D., 1992). In addition, the development pathways of transgene-expressing cells is not necessarily normal and caution must be exercised in interpreting results. Nevertheless, advent of new technologies will probably enable us to address this issue in a less altered environment.

Peripheral Tolerance

Although it is now well established that thymic clonal deletion is a major mechanism of tolerance for self antigens expressed in the thymus, the question as to how T cells are tolerized to self antigens encountered in extrathymic sites arises. It is difficult to conceive that *all* of the various self antigens of the extrathymic environment reach the thymus in sufficient quantities, and persist there long enough, to tolerize young T cells. What happens to self-reactive T lymphocytes that may escape thymic censorship or that may be reactive to unique extrathymic autoantigens? Since the manifestation of autoreactivity, *i.e.*, autoimmunity, is not the rule, it seems reasonable to assume that there exists peripheral mechanisms that induce T cell tolerance. From the available evidence, at least three peripheral mechanisms can be outlined: 1) functional silencing of T cells (anergy); 2) physical elimination of T cells having a strong reactivity to self antigens; 3) antagonism between T cell subsets with distinct lymphokine profiles, such as T_H1 and T_H2

(Immunoregulation/suppression).

Anergy

The recent description of tolerance in transgenic mice expressing MHC class I or II molecules on pancreatic β cells, and MHC class I on hepatocytes, strongly supports the concept of anergy. Lo and colleagues (Lo, D., Burkly, L.C., Widera, G., Cowing, C., *et al.* 1988) reported the presence of mature Vβ17a$^+$-TcR T cells in mice that expressed I-E class II MHC molecules on pancreatic islets cells. The integrity of the islets was maintained even when the mice were primed with I-E$^+$ spleen cells. The islets were rejected, however, if removed and grafted into I-E$^-$ mice and the recipient was primed with I-E$^+$ spleen cells (Markmann, J., Lo, D., Naji, A., Palmiter, R.D., *et al*, 1988). These observations demonstrate that the tolerance mechanism involves inactivation of the T cells in the transgenic mouse and not masking of the I-E molecules on the islet cells. MHC class I molecules on pancreatic β cells (Allison, J., Campbell, I.L., Morahan, G., Mandel, T.E., *et al.*, 1988; Morahan, G., Allison, J., and Miller J.F.A.P., 1989a) and on hepatocytes (Morahan, G., Brennan, F, Bhatal, P.S., Allison, J., *et al.*, 1989b) also induced peripheral T cell tolerance to the transgene-expressing organs and to class I-matching skin grafts but not to third party skin grafts. In addition, assays for specific anti-class I cytolytic T lymphocytes activity showed that thymocytes were able to respond to the transgenic class I whereas mature T cells were not.

The latter observation confirms the peripheral nature of this form of tolerance.

Since superantigens (superAgs, discussed in more details in chapter II) stimulate most T cells bearing a given TcR-Vβ-chain, several groups investigated the effects of intravenous injection of superAgs into normal adult mice. The injection of Mls-1^{a+} spleen cells into Mls-1^{b} (Mls-1^{a} negative genotype) mice induced an anergic state in the V$\beta6^{+}$/CD4^{+} (Mls-1^{a}-reactive) T cells (Rammensee, H.G., Kroschewski, R., and Frangoulis, B., 1989). Similar studies with an exogenous superAg, staphylococcal-enterotoxin B (SEB), also revealed an anergic state in Vβ-specific T cell populations (Rellahan, B.L., Jones, L.A., Kruisbeek, A.M., *et al.*, 1990; Kawabe, Y., and Ochi, A., 1990). The anergic state of the superAgs-tolerized T cells was manifested *in vitro* by lack of proliferation and of interleukin(IL)-2 (a T cell growth factor) production. Moreover, the addition of exogenous IL-2 *in vitro* did not counteract the nonresponsiveness of anergic cells.

SuperAg-induced anergic cells shared many of the characteristics described in an *in vitro* system of inducing anergy in T cell clones. In the case of IL-2-producing CD4^{+} T cell clones (T_H1 clones), occupancy of the TcR in the absence of a costimulatory signal from the APC induces a state of anergy (reviewed in Mueller, D.L., Jenkins, M.K., Schwartz, R.H., 1989). The requirement for this costimulatory signal is illustrated by the observation that T_H1 clones fail to proliferate or to produce IL-2 in response to peptide-pulsed, metabolically inactive

APCs or purified MHC molecules in artificial membranes. Subsequent challenge of these T_H1 clones with antigen and competent APC revealed that they were anergic. The costimulation of the T_H1 clones is provided by the engagement of their CD28 receptor with its ligand, B7, expressed on APCs (Jenkins, M.K., Taylor, P.S., Norton, S.D., Urdhal, K.B., 1991; Linsley, P.S., Brady, W., Grosmaire, L., Aruffo, A., *et al.*, 1991). However, the costimulus required may differ with the type of T helper clones used, as shown with some mouse IL-4-producing $CD4^+$ T cell clones (T_H2 clones) which depend on IL-1 for adequate activation (reviewed in Weaver, C.T., and Unanue, E.R., 1990). Pancreatic cells expressing a transgenic I-E molecule were unable to stimulate T lymphocytes reactive to I-E and, in fact, rendered the T cells anergic to subsequent stimulation by proper APCs (Markmann, J., Lo, D., Naji, A., Palmiter, R.D., *et al*, 1988). Thus, the theory that a lack of costimulation results in the induction of anergy has been tested *in vitro* in more than one system.

Although a lack of costimulation could account for some of the *in vivo* models of anergy induction, other biochemical defects seem to be involved in other systems. Anergic T cells from Mls-1^a mice expressing a TcR-Vβ8.1 transgene, another Mls-1^a specific Vβ-chain, showed a defect in Ca^{2+} signal transduction (Blackman. M.A., Finkel, T.H., Kappler, J., *et al.*, 1991). It was suggested that this form of anergy was caused by repeated TcR stimulation by the abundant antigen. *In vitro*, it was demonstrated that such chronic stimulation of T cell clones

eventually leads to TcR desensitization (Suzuki, G., Kawase, Y., Koyasu, S., Yahara, I., *et al*, 1988). Further studies will be required to delineate the biochemical defects found in anergic T cells, and to learn how to discriminate them in the various *in vivo* systems.

Peripheral Deletion

Peripheral T cell deletion has often been observed in association with cases of *in vivo* anergy induction. Injection of Mls-1^a (Webb, S., Morris, C., and Sprent, J., 1990) or SEB (Rellahan, B.L., Jones, L.A., Kruisbeek, A.M., *et al.*, 1990; Kawabe, Y., and Ochi, A., 1990) into mice generally provokes an expansion of T cells bearing reactive Vβ-TcR. This superAg-induced proliferation is followed by a sharp decline in superAg-reactive T cells. The deletion is partial, and as mentioned above, the non-deleted population of superAg-specific T cells is anergic. Thymectomy prior to exposure to superAgs did not alter the process, revealing that deletion was indeed a peripheral event (Webb, S., Morris, C., and Sprent, J., 1990; Jones, L.A., Chin, L.T., Longo, D.L., and Kruisbeeck, A.M., 1990; Kawabe, Y., and Ochi, A., 1991). Recently, we have demonstrated that partial peripheral deletion can occur in response to transplantation xenoantigens (see Chapter IV). By injecting rat spleen cells into C57BL/10 mice, we found that Vβ11$^+$ T cells were partially deleted. The nature of the ligand in this case is unknown.

How peripheral deletion is accomplished is not known, but there are some clues in the literature. Duke and Cohen (Duke, R.C., and Cohen, J.J., 1986) have shown that the removal of growth factors from activated T cells stops proliferation and leads to death by apoptosis. Conceivably, a rapid expansion of superAg-reactive T cells in an environment where the antigen concentration is allowed to decline below the level required for continued stimulation would cause T cell death by IL-2-deprivation. Another possibility is that the T cells are eliminated by veto cells. The veto cells are in fact cytolytic T lymphocytes that destroy other T cells whose TcR recognize antigens on the surface of veto cell (reviewed in Miller, R.G., 1986). The killing is thus independent of the specificity of the veto cell's TcR. Although this may explain the deletion of MHC class-I-restricted reactive T cells, it cannot apply to MHC class-II-restricted T cells, since murine T cells, including veto cells, do not express MHC class-II molecules. So it seems unlikely that superAgs, which bind to MHC class-II molecules, would support a veto phenomenon, at least in mice.

Immunoregulation/suppression

The first experimental data indicating that T cells could mediate an antagonistic (suppressor) effect came from the studies of Gershon and Kondo (Gershon, R.K., and Kondo, R.K., 1972). In some high antigen dose models, they showed that mixtures of nonresponsive and normal cell populations resulted in no suppression

response. This tolerance could be adoptively transferred to naive recipients with T cells and became known as 'infectious immunological tolerance'. The T cells transferring this tolerant state were termed T suppressor cells. These cells appeared to be part of a complex circuitry of various cell types with the overall function of negative feedback inhibition of the immune response (reviewed in Germain, R.N., and Benacerraf, B., 1981). However, to this date, the identity and cloning of so-called 'suppressor T cells' has remained elusive (Möller, G., 1988). A reexamination of the literature suggests that in most cases, the suppressor phenomenon is explainable by the effects of cytokines or killing by cytotoxic $CD8^+$ T cells (veto cell concept, as discussed above). The delineation made by Mossman and colleagues (reviewed in Mosmann, T.R., and Coffman,R. L., 1989) of T_H1 and T_H2 murine $CD4^+$ T cell clones, which differ in their lymphokine secretion pattern, revealed that they were antagonistic to each other. T_H1 clones secrete IL-2, lymphotoxin-β (TNF-β), and interferon-γ (INF-γ) which mediate delayed-type hypersensitivity (DTH) responses, whereas T_H2 clones produce IL-4, IL-5, IL-6, and IL-10 which promote antibody production. It was shown that INF-γ secreted by T_H1 clones inhibits the proliferation and function of T_H2 clones (Fernandez-Botran, R., Sanders, V.M., *et al.*, 1988), while the T_H2-derived IL-10 produces the same suppressive effect on T_H1 clones indirectly by acting on APCs (Mosmann, T.R., Schumacher, J.H., Street, N.F., *et al.*, 1991). Thus, some early suppression studies may be examples of T_H1/T_H2 interregulatory effects. For

example, the phenomenon of low dose nonresponsiveness at the level of antibody production, first reported by Mitchison (Mitchison, N.A., 1964), was widely interpreted in the 1970's as evidence of suppressor T cell activity. However, when Parish and Liew (Parish,C.R., and Liew, F.Y., 1972) repeated the experiments, they found that despite the low levels of antibody production, a strong DTH response could be detected. In light of more recent studies, their observations are compatible with T_H1/T_H2 antagonistic regulation. Examples of T_H1/T_H2 inhibition were also reported in parasitic infections of various mouse strains (Locksley, R.M., Heinzel, F.P., Sadick, M.D., *et al.*, 1987). Although certain immune responses appear to be dominated by the activities of one of the two subsets, it is still not clear if T cell subpopulations corresponding to T_H1 or T_H2 clones develop *in vivo*.

2. Cyclosporin A

The discovery of cyclosporin A (CsA) has revolutionized transplantation medicine. The pre-cyclosporin era of organ transplantation was impeded by the high rejection rates and by the serious side effects of the contemporary immunosuppressive drugs. With the advent of CsA, allograft survival greatly improved, and its side effects were far less serious than those of its predecessors. Nowadays, an estimated 150 000 transplant patients worldwide are relying on CsA (Borel, J.F. and Kis, Z.L., 1991), and some promising results are obtained in treatment of autoimmune diseases (reviewed in Bach, J.F., 1993). In addition to its successful use in clinical practices, CsA is also widely employed as an experimental tool for basic research in signal transduction pathways (reviewed in Sigal, N.H., and Dumont, F.J., 1992; and Liu, J., 1993). Despite the enormous amount of studies involving CsA (over 20 000 publications since 1976), the mechanism of action of CsA is still uncertain. Furthermore, CsA has also been shown to create unexpected and paradoxical immunological conditions. For example, CsA can induce long-term transplantation tolerance that does not require the continuation of immunosuppressive therapy, but sometimes paradoxically induces autoimmune diseases (reviewed in Prud'homme, G.J., and Vanier, L.E., 1993 [Appendix B]). Attempts have been made to understand how CsA induced these conditions by studying its effects on thymic functions. Although some thymic

dysfunctions were found (reviewed in Prud'homme, G.J., Parfrey, N.A., and Vanier, L.E. 1991a [Appendix A]), it remains unclear that these abnormalities were responsible for the CsA-induced autoimmunity. In addition, previous studies have not addressed the effects of CsA on peripheral tolerance. Moreover, no *in vivo* study has been done on peripheral deletion and anergy during CsA-treatment. This is what prompted my studies of the effects of CsA on peripheral tolerance. But first, we will briefly review our current knowledge on CsA.

Discovery and Clinical Relevancy of CsA

CsA was isolated in 1970 from a new strain of fungus, *Tolypocadium inflatum*, that was grown out from a Norwegian soil sample. First noted for its antifungal activity, It turned out that CsA possessed only a narrow spectrum of antifungal activity *in vitro* and a marginal one *in vivo*; the latter being mainly against relatively innocuous strains such as *Candida albicans* (Borel, J.F. and Kis, Z.L., 1991). However, it was observed that CsA had an unusually low degree of toxicity. This particular observation incited researchers at Sandoz to pursue further pharmacological screening. The immunosuppressive potential of the drug was discovered by Borel and colleagues in 1972. Using hemagglutination tests after *in*

vivo immunization of mice with sheep red blood cells, they found that CsA had a potent immunosuppressive effect. Furthermore, they observed that CsA would not prolong the survival time of mice injected with a leukemic cell line (L-1210), nor would it affect the growth of murine tumor cells, P-815, *in vitro* (Borel, J.F., 1982). This clearly established that, unlike the immunosuppressants of the time, CsA's immunosuppression was not linked with a general cytostatic activity. Henceforth, CsA extracts were purified, and chemical structure analysis was performed (Petcher, T.J., Weber, H.P., and Rüegger, A, 1976). These studies revealed that the most potent molecule isolated, then termed CsA, was a neutral, lipophilic, and cyclic endecapeptide, with a molecular weight of 1203.

Borel and colleagues reported their findings on CsA in 1976 (Borel, J.F., Feurer, C., Gubler, H.U., and Stähelin, H, 1976). They demonstrated that CsA had a selective and reversible inhibitory effect on helper T cells, was non-mutagenic, and had no myelotoxicity. CsA was then successfully tested in several species for its ability to prolong the survival of various organ allografts (Green, C.J., and Allison, A.C., 1978; Calne, R.Y., White, D.J.G., Rolles, K., *et al.*, 1978a). The first human trials were equally promising. Transplantation of mismatched cadaver kidneys (Calne, R.Y., White, D.J.G., Thiru, S., *et al.*, 1978b), livers, and pancreases (Calne, R.Y., Rolles, K., White, D.J.G., *et al.*, 1979) proved the efficiency of CsA in preventing human allograft rejections. These studies also confirmed the clinical advantages of CsA over the conventional

immunosuppressive drugs. As observed in animal studies, and unlike azathioprine, methotrexate or cyclophosphamide, CsA had a minimal effect on hematopoiesis, and contrarily to glucocorticoids, it did not inhibit macrophage and neutrophil functions (Feutren, G., 1992a). However, CsA therapy was not without any side effects (reviewed in Kahan, B.D., 1992). Some were transient and relatively benign like hypertension, light gastrointestinal complications, tremor, etc... But the most limiting side effect was, and remains, kidney dysfunction (Mihatsch, M.J., 1992). Based on the original recommendation of Starzl (Starzl, T.E., Hakala, T.R., Iwatsuki, S., *et al.*, 1982), CsA is now used in combination with other immunosuppressants, especially prednisone. This was found to alleviate some of the side effects caused by either drug, since smaller doses could be used (Kahan, B.D., 1992).

CsA has dramatically improved clinical transplantation, particularly situations with greater risks of lethal rejection episodes, such as cardiac, liver, heart-lung, and multiple organ transplantation. CsA has also been used with some degree of success to treat several autoimmune diseases such as psoriasis, Behçet's disease, and insulin-dependent type-I diabetes (reviewed in Feutren, G., 1992b).

Mechanisms of action

The selective immunosuppressive properties of CsA are believed to result from the inhibition of transcription of lymphokines, most notably IL-2, the main growth factor of T cells (Elliot, J.F., Lin, Y., Mizel, S.B., *et al.*, 1984; Krönke, M., Leonard, W.J., Depper, J.M., *et al.*, 1984). Since IL-2 synthesis is generally associated with a successful T cell activation, CsA provided an opportunity for probing the intracellular signalling pathways of T cells. CsA was found to bind an ubiquitous and abundant family of cytosolic proteins, cyclophilins, which all shared a *cis-trans* peptidyl-prolyl isomerase activity (also known as a rotamase activity) that was inhibited upon binding with CsA (Handschumacher, R.E., Harding, M.W., Rice, J., *et al.*, 1984; Fisher, G., Wittmann, L.B., Lang, K., *et al.*, 1989; Takahashi, N., Hayano, T., and Suzuki, M., 1989). Studies with CsA analogues demonstrated that the immunosuppressive activity was dependent on cyclophilin binding (Quesniaux, V.F., Schreier, M.H., Wenger, R.M., *et al.*, 1987). The interest in a potential role of rotamases in T cell activation pathways greatly increased when it was found that FK-506, another fungal compound with similar spectrum of activity to CsA but structurally different, was also binding a distinct family of proteins (FK-506-binding protein, FKBP) with peptidyl-prolyl isomerase activity (Sierkierka, J.J., Hung, S.H., Poe, M., *et al.*, 1989; Harding, M.W.,

Galat, A., Uehling, D.E., and Schreiber, S.L., 1989). This lead to a series of studies on the rotamase activity of these cellular receptors, which are now referred to as immunophilins (reviewed in Schreiber, S.L., 1991). However, evidence against the role of rotamase activity in immunosuppression soon accumulated. Beside the fact that no protein has thus far been shown to depend on immunophilins for its folding, only a fraction of immunophilins were bound to CsA or FK-506 at optimal concentration of these drugs (Sigal, N.H., and Dumont, F.J., 1992). Moreover, analogues of CsA and FK-506 showed no correlation with the inhibition of rotamase activity and their immunosuppressive properties (Sigal, N.H., Dumont, F., Durette, P., *et al.*, 1991; Bierer, B.E., Somers, P.K. Wandless, *et al.*, 1990; Dumont, F.J., Staruch, M.J., Koprak, S.L., *et al.*, 1992). The questions raised by these discrepancies were answered with the discovery of a common target for both drug/immunophilin complexes: calcineurin (Friedman, J., and Weissman, I., 1991; Liu, J., Farmer, J.D.Jr., Lane, W.S., *et al.*, 1991). Calcineurin is a Ca^{2+}-and calmodulin-dependent serine/threonine phosphatase composed of a catalytic (A) and regulatory (B) subunits (reviewed in Klee, C.B., Draetta, G.F., and Hubbard, M.J., 1988). Surprisingly, despite the fact that there are no obvious structural similarities in the two drug/immunophilin complexes, they competitively bind to a common site on calcineurin. The binding of the drug/immunophilin complexes with calcineurin inhibits the phosphatase activity of the latter. In addition, non-immunosuppressive analogues exhibit very weak

inhibitory activity toward calcineurin (Liu, J., Albers, M.W., Wandless, T.J., *et al.*, 1992). Recent *in vitro* evidence shows that calcineurin is a rate-limiting enzyme of the signalling pathway for IL-2 transcription (Clipstone, N.A., Crabtree, G.R., 1992; O'Keefe, S.J., Tamura, J., Kincaid, R.L., *et al.*, 1992). Henceforth, calcineurin was shown to be linked to a transnuclear signalling pathway regulating IL-2 synthesis (McCaffrey, P.G., Perrino, B.A., Soderling, T.R., and Rao, A., 1993). It would appear that the nuclear factor of activated T cells (NF-AT), a transcription factor exclusive to T cells, is a substrate of calcineurin. NF-AT is composed of two subunits, a constitutively expressed cytosolic phosphoprotein, NF-ATc, and a nuclear protein kinase C (PKC)-inducible component, NF-ATn (Flanagan, W.F., Corthésy, B., Bram, B.J., and Crabtree, G.R., 1991; Jain, J., McCaffrey, P.G., Valge-Archer, V.E., and Rao, A., 1992). A functional transcription factor is obtained by the Ca^{2+}-dependent translocation of NF-ATc into the nucleus, so that both subunits can combine. Both the translocation event and the dephosphorylation of NF-ATc by calcineurin are inhibited by a calcium chelator (namely EGTA), and by pretreatment of the cells with CsA or FK-506 (Flanagan, W.F., Corthésy, B., Bram, B.J., and Crabtree, G.R., 1991; McCaffrey, P.G., Perrino, B.A., Soderling, T.R., and Rao, A., 1993). Although dephosphorylation and translocation of NF-ATc has not been demonstrated to be consequent yet, this is the closest we have come in integrating the calcineurin activation pathway to the NF-AT transcription events (Fig. 1).

Lastly, some data suggest the possibility of a CsA surface receptor, possibly a member of the cyclophilin family, that would convey the immunosuppressive activities of CsA (reviewed in Erlanger, B.F., 1992). However, it should be noted that these results do not invalidate the concepts described above, since some calcineurin activity has also been reported to be associated with the membrane of T cells (Alexander, D.R., Hexham, J.M., and Crumpton, M.J., 1988).

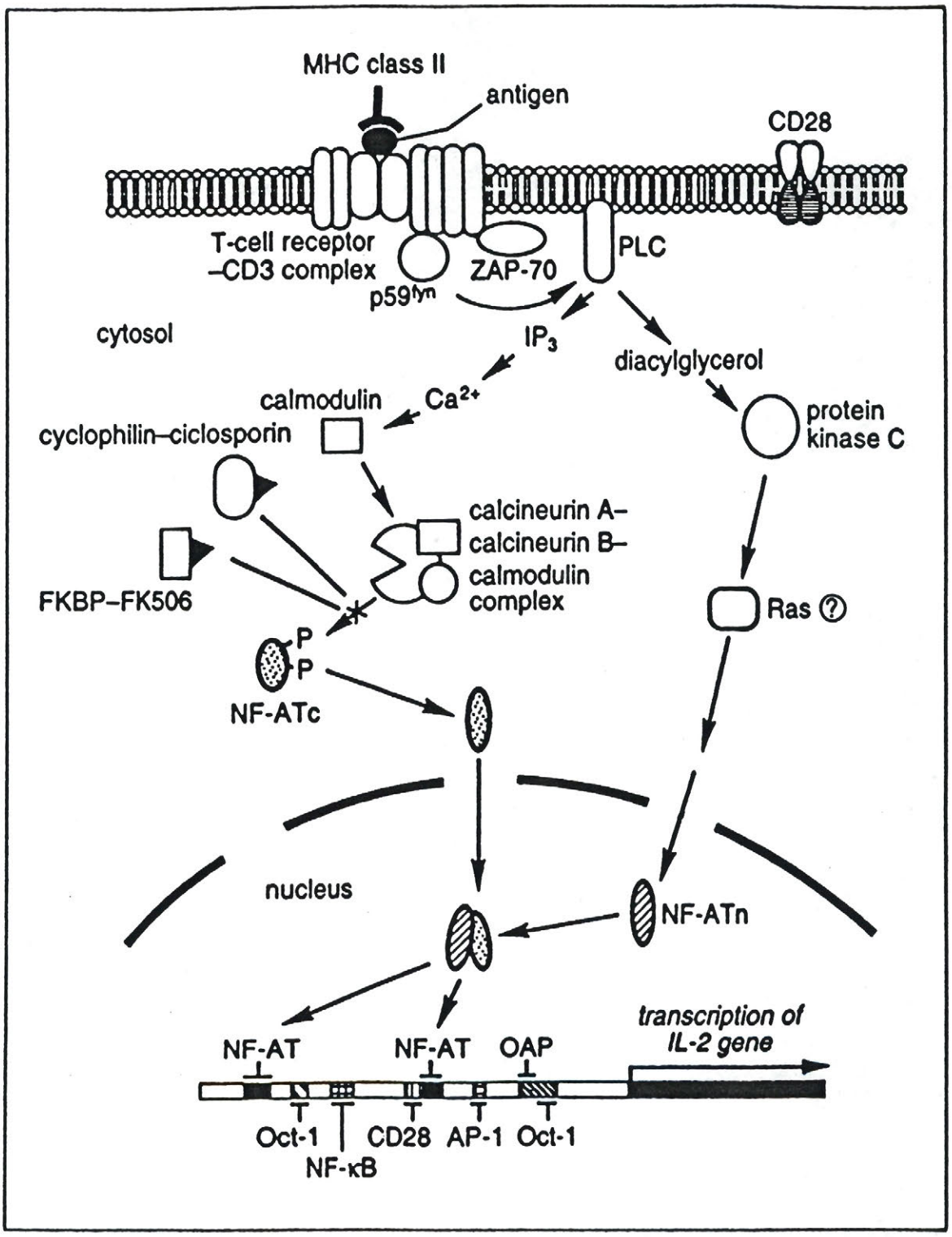

Fig. 1 *The T-cell receptor-mediated signal transduction pathway leading to interleukin 2 (IL-2) transcription with the recently identified signal transducing molecules highlighted.* PLC: *phospholipase Cγ1;* IP_3: *inositol-1,4,5-trisphosphate;* NF-ATc: *the cytoplasmic component of the nuclear factor of activated T cells;* NF-ATn: *the nuclear component of NF-AT;* FKBP: *FK506 binding protein.* (LIU, J. 1993)

3. Effects of CsA on Tolerance Mechanisms

As we have seen, the current concept for CsA's activity is the inhibition of IL-2 synthesis. However, some immunological conditions caused by CsA suggest that it may interfere with several T cell tolerance mechanisms (reviewed in Prud'homme, G.J., and Vanier, L.E., 1993 [Appendix B]). CsA can enhance tolerance mechanisms, as in nonimmunosuppressed long-term allograft tolerance, where after a short course of CsA-treatment the graft is permanently accepted by the host. But CsA can also disrupt immune tolerance, like in CsA-induced syngeneic graft-versus-host disease (GVHD) (reviewed in Prud'homme, G.J., Parfrey, N.A., and Vanier, L.E. 1991a [Appendix A]). What causes the occurrence of these paradoxical effects is still not fully understood, but several studies have provided some insights on some tolerance mechanisms affected by CsA.

Effects of CsA on Thymic Events

Since the study of Sorokin and co-workers (Sorokin, R., Kimura, H., Schroder, K., Wilson, D.H., and, Wilson, D.B., 1986), we know that the presence of a thymus is imperative for the induction of syngeneic GVHD. The first thymic abnormality attributed to CsA was atrophy of the thymic medulla (Baldwin, W.M., Hutchison, I.F., Meijer, J.L.M., and Tilney, N.L., 1981). The atrophy resulted

from an arrest of thymocyte development at the DP stage (Jenkins, M.K., Schwartz, R.H., and Pardoll, D.M., 1988a; Gao, E.-K., Lo, D., Cheney, R., *et al.*, 1988). However, the blockage of thymocyte development was not complete, and some of the few maturing thymocytes expressed self-reactive TcRs. In addition, Shi and colleagues (Shi, Y., Sahai, B.M., and Green, D.R., 1989) reported that CsA prevents apoptosis in thymocytes. These observations suggest that the negative selection process is impaired. The abrogation of the thymic clonal deletion of self-reactive T cells implied that the non-deleted T cells could seed the periphery and induce GVHD. Syngeneic GVHD was reported in some murine strains where the animals were irradiated and injected with CsA for a relatively short period of time, usually a few weeks (Bryson, J.S., Jennings, C.D., Caywood, B.E., and Kaplan, A.M., 1989). The first symptoms of GVHD-like disease generally appeared within a week after cessation of CsA injections. Disease cannot be easily attributed to a defect in clonal deletion since no correlation with the presence of self-reactive T cells and the induction of syngeneic GVHD has been found (Prud'homme, G.J., Sander, R., Parfrey, N.A., and SteCroix, H, 1991b; Bryson, J.S., Carwood, B.E., and Kaplan, A.M., 1991). In fact, T cells bearing self-reactive TcRs could be detected in murine strains resistant to syngeneic GVHD, and not detected in sensitive strains (Bryson, J.S., Carwood, B.E., and Kaplan, A.M., 1991). In addition, recent observations made in TcR-transgenic mice have shown that CsA delays, but does not abolish, clonal deletion

(Urdahl, K.B., Pardoll, D.M., and Jenkins, M.K., 1994). These discrepancies do not necessarily exclude the involvement of thymic negative selection in syngeneic GVHD, but they suggest a more complex process, possibly the contribution of peripheral tolerance mechanisms.

CsA can also induce an organ-specific form of autoimmunity. The injection of CsA in newborn mice for a week subsequently produces several organ-specific autoimmune diseases, including gastritis, thyroiditis, insulitis, oophoritis and others, all accompanied by organ-specific autoantibodies (Sakaguchi, S., and Sakaguchi, N., 1989). The development of autoimmune diseases can be prevented by injection of splenic T cells from syngeneic normal mice. The thymus was shown to play a key role in the development of organ specific autoimmune diseases. Thymic grafts of CsA-treated newborn or adult mice into syngeneic athymic nude (nu/nu) mice produced the same profile of organ-specific lesions reported in CsA-treated newborn mice (Sakaguchi, S., and Sakaguchi, N., 1988). Co-transplantation of CsA-treated and normal thymi counteract the induction of organ-specific autoimmune diseases in nude mice. These findings are reminiscent of studies where T cell population imbalances created immunoregulatory defects. Adult thymectomy or anti-thymocyte serum treatments were shown to enrich lymphoid organs with a long-lived T helper population having a T_H2-profile of cytokine secretion (Swain, S.L., McKenzie, D.T., Dutton, R.W., *et al.*, 1988). Conceivably, the permissiveness of the CsA-treated thymus may enrich the

periphery with autoreactive T_H2-like T cells at the expense of the short-lived T_H1-like T cells. Thus, the reinsertion in autoimmune-prone mice of mature T cells, or of a normal thymus might reestablish the balance between T helper populations and consequently, prevent the induction of autoimmune lesions. This proposal remains speculative since no study was performed on T_H1/T_H2 imbalance in mice affected with organ-specific autoimmune diseases.

Experimental models of CsA-induced syngeneic GVHD and organ-specific autoimmune diseases have clearly demonstrated that the disruption of thymic events was a required step in the pathogenesis of these diseases. However, the discrepancies found between the detection of self-reactive T cells and the expression of GVHD, as well as the ability of normal syngeneic T cells to prevent autoimmune disease, all suggest that CsA must affect some peripheral tolerance mechanism as well.

Effects of CsA on Peripheral Tolerance

The effects of CsA on clonal anergy were only investigated *in vitro* with $CD4^+/T_H1$ T cell clones. Jenkins and colleagues (Jenkins, M.K., Ashwell, J.D. and Schwartz, R.H., 1988) have shown that CsA prevents the induction of anergy in these clones. How CsA blocks anergy is still unknown, but based on our current knowledge of anergy induction in these clones, we can offer an hypothesis. Full activation of T_H1-clones requires two signals, *i.e.*, a TcR-mediated signal, and a

costimulatory signal transmitted by the CD28 surface receptor (reviewed in Schwartz, R.H., 1990). Triggering of the TcR-mediated signal alone will induce anergy (see previous section on anergy). Since the CD28 costimulatory signal is not affected by CsA (June, C.H., Ledbetter, J.A., Gillespie, M.M., *et al.*, 1987; Bjorndahl, J.M., Sung, S.S.,Hansen, J.A., and Fu, S.M., 1989; Hess, A.D., Bright, E.C., 1991; Jenkins, M.K., Taylor, P.S., Norton, S.D., and Urdahl, K.B., 1991), CsA is likely to block anergy induction by interfering with the TcR-mediated signal. This is further supported by the observation that both the TcR-mediated signal and anergy are Ca^{2+}-dependent.

CsA was shown in many species to induce enhanced allograft tolerance after a short course of CsA-treatment (reviewed in Green, C.J., 1988). The grafts were accepted permanently, or at least for a prolonged period of time, by the host without further supplementation of immunosuppressants. Tolerance to allografts could be adoptively transfered by injection of $CD4^+$ T cells, implying a T helper-immunoregulatory mechanism of tolerance (Hall., B.M., Pearce, N.W., Gurley, K.E., and Dorsch, S.E., 1990). However, more recent work by Miyagawa and colleagues (Miyagawa, S., Lawen, J.G., Stepkowski, S.M., Kahan, B.D., 1991) suggests that deletion of alloantigen-specific T cells contributes to the prolonged survival of allografts. Unfortunately, the technique these investigators used (limiting dilution assays) could not distinguish between anergy and deletion or even specific T cell suppression. Physical evidence of deletion, such as phenotypic

analysis, is definitely required for a better understanding of CsA-enhanced allograft tolerance phenomenon.

The effects of CsA on *in vivo* T_H1/T_H2 immunoregulatory pathways are contradictory. Studies with the parasitic pathogen *Leishmania major* in Balb/c mice have shown that CsA confers a resistance to *L. major* by enhancing T_H1-dependent DTH responses (Behforouz, N.C., Weryer, C.D., and Mathison, B.A., 1986). T_H1-dependent DTH responses were also promoted by CsA in response to sheep red blood cells (Webster, L.M., and Thompson, A.W., 1988). In both of these experiments a T_H1-shift is inferred from the marked reduction of antibody production (T_H2-dependent) against the antigens used. In contrast, Chen *et al.* (Chen, S.S., Stanescu, G., Magalski, A.E., and Qiang, Y.Y., 1989) found that injection of CsA before antigen priming potentiates the T_H2-dependent IgE antibody production. *In vitro* studies also suggest that T_H2 clones are more resistant to CsA than T_H1 clones (Gajewski, T.F., Schell, S.R., and Fitch, F.W., 1990). Interestingly, recent findings demonstrate that CsA can alternatively favor T_H1-like responses if the antigen administration was designed for a T_H2-like stimulation, or T_H2-like responses when antigen administration should have promoted a T_H1-like stimulation (Bretscher, P.A., and Hasele, C., 1992). Why CsA has the tendency to support the opposite immune response than the one originally expected is not known.

It is essential that we learn more of the effects of CsA on peripheral tolerance mechanisms. Although an adequate study of T_H1/T_H2 activity and antagonism is difficult to achieve due to the lack of phenotypic markers, peripheral deletion and anergy can now be studied *in vivo* through the use of superAgs. This is why I undertook to study the effects of CsA on peripheral deletion and anergy. Such studies will provide a better understanding as to how CsA affects peripheral tolerance, and might eventually lead to improved management of CsA therapy, particularly to avoid CsA-induced autoimmune diseases, or even to promote enhanced allograft tolerance.

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CHAPTER II.

STUDIES ON PERIPHERAL DELETION AND ANERGY

INDUCED BY SUPERANTIGENS

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Submitted for Publication

1. Introduction

Superantigens (superAgs) are products of bacteria and viruses which can stimulate a large fraction of mature T cells (reviewed in Drake, C.G., and Kotzin, B.L., 1992; Hodes, R.J., and Abe, R., 1992; Johnson, H.M., Russell, J.K., and Pontzer, C.H., 1991). The basis of their potent stimulatory effects lies with their ability to bind the relatively limited numbers of Vβ segments of the T cell receptor (TcR). Thus, depending on the frequency of the responding Vβ subpopulation(s), 5 to 30% of the entire T cell repertoire can be stimulated by a given superAg. SuperAgs do not require processing by the antigen presenting cells (APCs), since they interact directly with amino acid residues located outside of the conventional peptide binding groove of MHC class II molecules. A schematic representation of the particular TcR-MHC interaction is shown in figure 1. SuperAgs are ideal tools for probing T cell tolerance phenomena. We can easily assess functional tolerance by measuring *in vitro* responses by virtue of their strong mitogenic properties, and their limited TcR specificity enables us to monitor the proportion of T cells bearing the relevant Vβ-TcR by flow cytometric analysis. In this study, we have used both the exogenous and endogenous forms of superAgs. The exogenous and the endogenous superAgs were respectively the *Staphylococcus aureus* enterotoxin B (SEB), and the product of the intrinsic mouse mammary tumor virus 7 (MMTV-7), more commonly referred to as Mls-1^a. SEB stimulates T cells

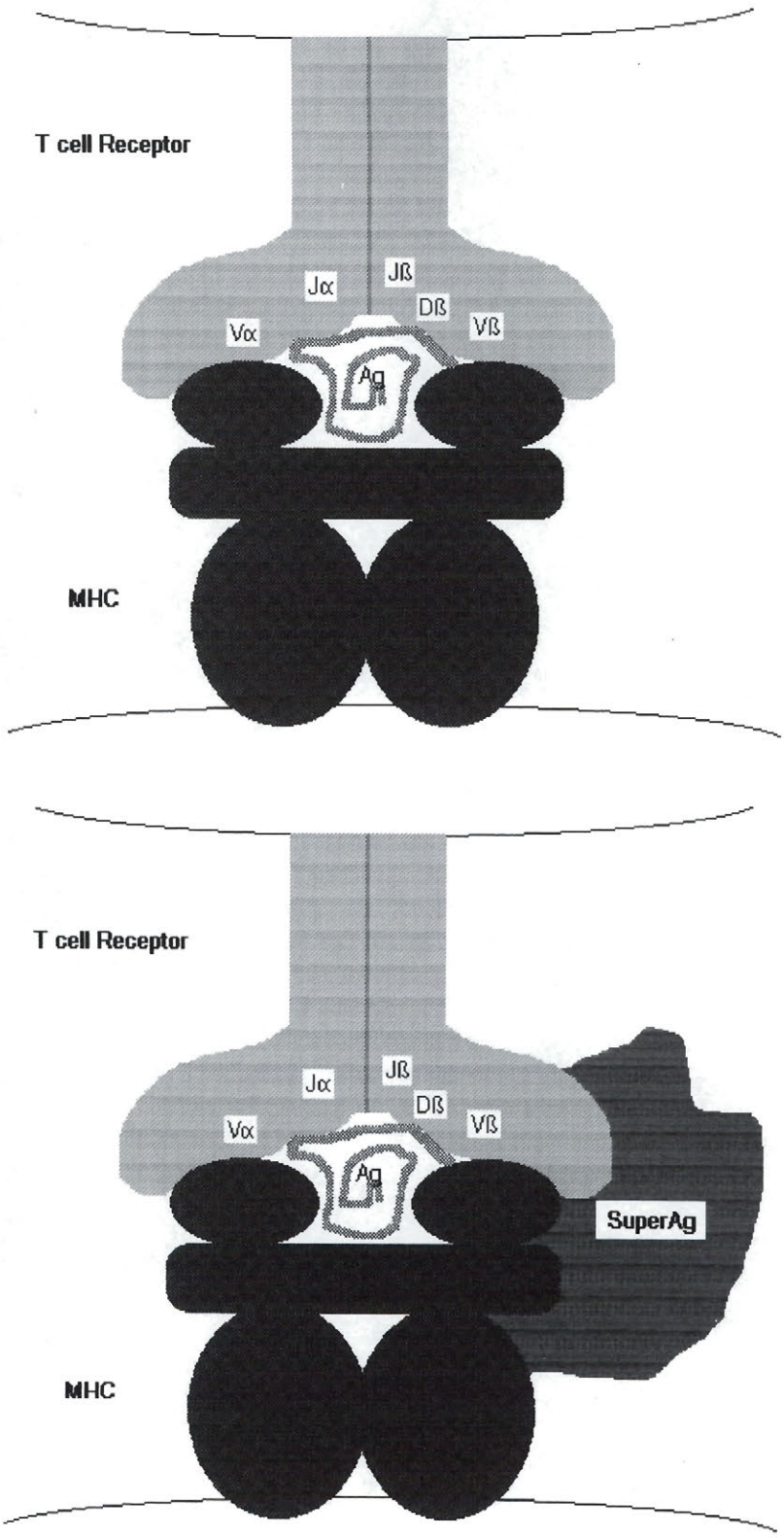


Figure 1. (A) Schematic representation of the αß TcR complexed to MHC molecule (α helices and ß-pleated sheet represented by the circles and the platform respectively) and antigen (Ag). (B) Representation of superantigen (superAg; staphylococcal enterotoxins and Mls) interactions with TcR-MHC complex.

bearing Vβ3, 7, 8, 11, and 17 segments, whereas the Mls-1^a molecule triggers the activation of T cells bearing Vß6, 7, 8.1, and 9 segments. However, due to the relative frequencies of the Vß expressing T cell populations in most murine strains, the observed effects of SEB are generally caused by the activation of Vß8$^+$/CD4$^+$ T cells, and Mls-1^a by the Vß6$^+$/CD4$^+$ T cells. When injected intravenously to mice, superAgs induce anergy as well as partial peripheral deletion of CD4$^+$ T cells bearing superAgs-specific TcR-Vβ segments (Rammensee, H.G., Kroschewski, R., and Frangoulis, B., 1989; Rellahan, B.L., Jones, L.A., Kruisbeek, A.M., *et al.*, 1990; Kawabe, Y., and Ochi, A., 1990; Vanier, L.E. and Prud'homme, G.J. 1992 [chapter III]). The current study is aimed at defining the characteristics and nature of T cell anergy and peripheral deletion; it includes *in vivo* kinetic studies, T cell growth factor requirements, effects of thymectomy, dose of superAg used for immunization, and the possible contribution of suppressor factors in maintaining anergy.

2. Materials and Methods

Mice

BALB/c mice (H-2^d, Mls-1^b, Mls-2^a, Lyt-1.2, Lyt-2.2), DBA/2 mice (H-2^d, Mls-1^a, Mls-2^a, Lyt-1.1, Lyt-2.1) were purchased from Charles River Laboratory (St-Constant, Quebec, Canada), and C3H/HeJ mice (H-2^k, Mls-1^b, Mls-2^a) from the Jackson Laboratory (Bar Harbor, ME). Adult BALB/c mice (4 to 6 weeks old) were thymectomized in our facility as described (Herbert, W.J., and Kristensen, F., 1986) and rested for 7 days before the onset of treatments. All the experiments were performed on female mice.

MoAbs and Reagents

Phycoerythrin(PE)-conjugated anti-CD4, PE-conjugated anti-CD8, anti-Lyt-1.1, anti-Lyt-2.1, and anti-Thy-1.2 were purchased from Cedarlane (Hornby, Canada). The following MoAb's were produced as culture supernatants and purified by protein G (Pharmacia, Montreal, Quebec, Canada) affinity chromatography: anti-Vβ6 (44-22-1 hybridoma [Payne, J., Huber, B.T., Cannon, N.A., Schneider, R., *et al.*, 1988]), anti-Vβ8.1-8.2 (KJ16 hybridoma [Haskins, K., Hannum, C., White, J., Roehm, N., *et al.*, 1984]), anti-Vβ14 (14.2 hybridoma [Liao, N.-S., Maltzman, J., and Raulet, D.H., 1989]), and anti-TcR-$\alpha\beta$ (H57-579 hybridoma [Kubo, R.T., Born, W., Kappler, J.W., *et al.*, 1989]). For flow cytometry

analysis the purified MoAbs were directly conjugated with fluorescein isothiocyanate (FITC). CsA was a kind gift of Sandoz (Dorval, Canada). SEA was purchased from Toxin Technology (Sarasota, FL) and SEB was purchased from Sigma Chemical Co. (St-Louis, MO).

SuperAg-immunization

SuperAg immunizations were performed by tail vein injections of either 10^7 Mls-1^{a+} splenocytes (RBC depleted) or 50-100μg of SEB. Cells or SEB were injected in a volume of 0.25ml of PBS. The day of superAg injection was considered day 0 of our experiments.

Flow cytometry analysis

For two color flow cytometry analysis, lymph nodes (LN) cells (pooled cervical, axillary, para-aortic and mesenteric LN) were stained with either PE-anti-CD4 or PE-anti-CD8 and with one of the FITC-labelled-anti-Vβ MoAbs mentioned above. One color flow cytometry analysis was performed with FITC-labelled MoAbs only. Briefly, $5x10^5$ LN cells were incubated with PE-conjugated MoAb for 15 minutes at 4°C, then washed 3 times with PBS containing 1% FCS (Gibco, Burlington, Canada), and then incubated in the same conditions with the second FITC-labelled-anti-Vβ MoAb. Propidium iodide (Sigma Chemical Co.) was added after the last wash. Dead cells were excluded based on propidium iodide staining

and forward scatter. In each sample, 10^4 cells were analyzed on a FACScan (Becton and Dickenson, Missisauga, Canada) flow cytometer, and results were plotted on a logarithmic scale. In some experiments spleen cells were analyzed. Before staining, spleen cells (RBC depleted) were depleted of B cells by two rounds of sequential J11.D2 MoAb and complement treatment, performed as we have previously described (Prud'homme, G.J., Bocarro, D.C., and Luke, E.C.H., 1991). Following depletion of dead cells on a lympholyte-M gradient (Cedarlane), spleen cells were stained and analyzed as described above for LN cells.

In vitro proliferative assays

LN cells or purified-T cells were cultured in 96 flat bottom plates (Gibco) in quadruplicates at $5x10^5$ cells/well with either: a) for SEB stimulation, with 10^6 syngeneic irradiated (2000 rads) splenocytes of untreated BALB/c mice and 10μg/ml of SEB, or 1μg/ml of SEA as a control; or b) for Mls-1^a experiments, with 10^6 irradiated (1000 rads) DBA/2 splenocytes or irradiated (1000 rads) allogeneic C3H/HeJ (H-2^k, Mls-1^b) splenocytes. Cells were cultured in RPMI-1640 (Gibco) containing 10% FCS, L-glutamine, $5x10^{-5}$M 2-mercaptoethanol and antibiotics. Cells were pulsed with [^{3}H]-thymidine (ICN, Montreal, Canada) on day 3 of culture and harvested on day 4, unless otherwise indicated. For experiments that assessed the effect of exogenous IL-2 or MLC supernatant additions, RIL-2 (Cedarlane, Hornby, Canada) and MLC supernatant (taken from

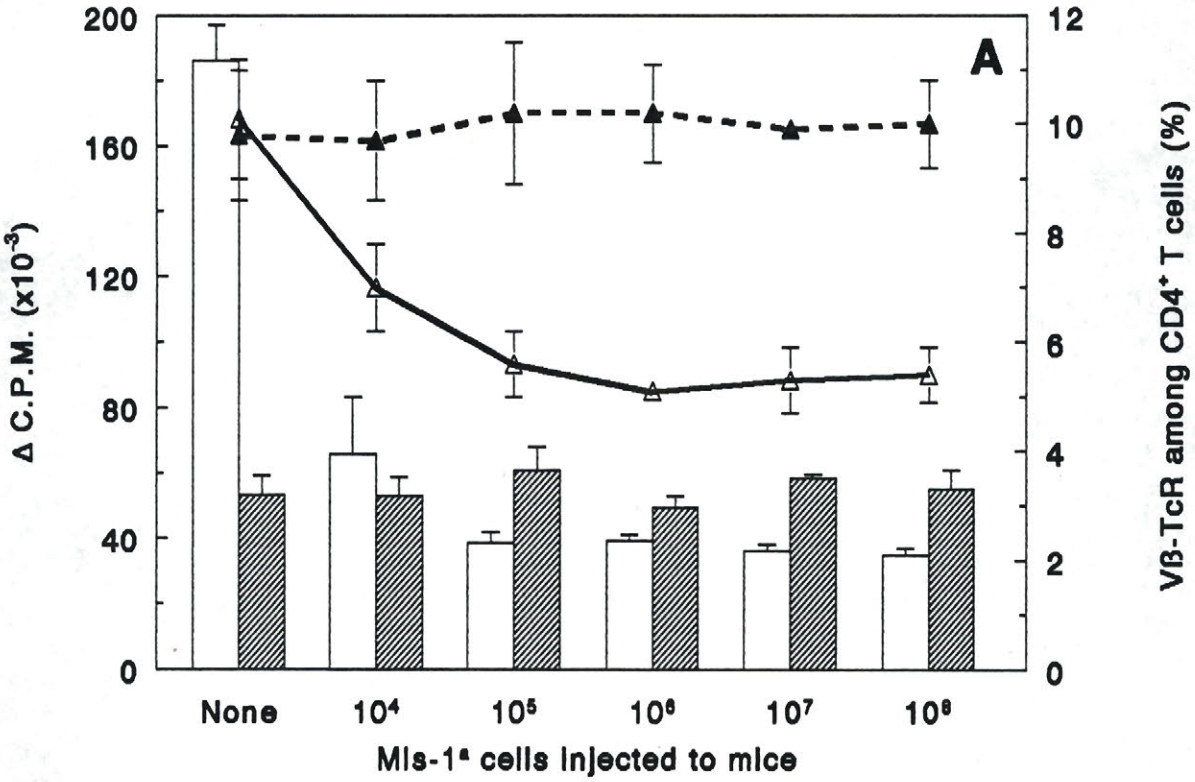
cultures of BALB/c T cell responders with CBA/J splenocytes) were added in culture wells at the beginning of the proliferation assay. For experiments where responder T cells were mixed in the same culture well, the T cells were purified by passage through a cellect^{tm} T cell purification column (Biotex Laboratories Inc., Edmonton, Canada) before being added to culture wells. Day in culture refer to the day that the cells were harvested. In all cases, the cells were harvested after 18 hours of incubation with [^{3}H]-thymidine. In the case of stimulation with either SEA or SEB, Δcpm = (cpm experimental) - (cpm control [no superAg]). In the case of Mls-1^a or MLC stimulation, Δcpm = (cpm experimental) - (cpm with syngeneic stimulator cells).

3. Results

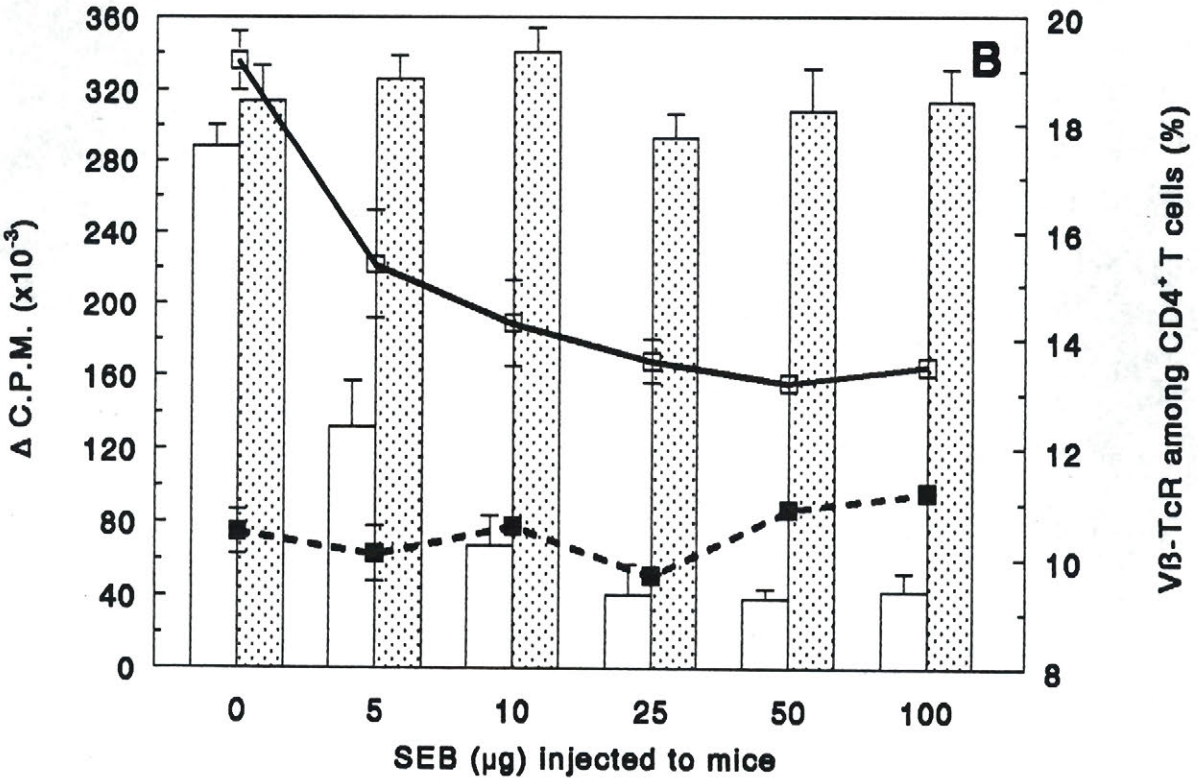
Effects of dose and repeated superAg-injections on superAg-reactive T cells. SuperAgs are potent inducers of anergy as demonstrated in Fig. 2. *In vitro* proliferative assays indicate a 5 to 7 fold reduction in reactivity to the superAg used to immunize the mice. The extent of the decrease in proliferative responses to superAg in anergic T cells reached a maximum at a dose of 10^5 Mls-1^{a+} cells and 25μg of SEB, in Mls-1^a-injected and SEB-injected mice, respectively. The extent of the partial deletion of superAg-reactive T cells in these mice also reached a plateau at the same doses. Both anergy and partial deletion are specific to the superAg-reactive T cell populations. The non-Mls-1^a-reactive T cells ($CD4^+/V\beta14^+$ T cells) showed no significant variation in their relative frequencies in Mls-1^a-injected BALB/c (Mls-1^b; H-2^d) mice, and the *in vitro* proliferative responses to irradiated allogeneic C3H/HeJ (Mls-1^b; H-2^k) spleen cells (MLC stimulator) remained comparable to PBS-injected BALB/c mice (Fig. 2a). Likewise, non-SEB-reactive T cells ($CD4^+/V\beta6^+$ T cells) in SEB-injected mice showed no reduction in their numbers (not shown), but their relative frequencies were slightly increased, which is likely due to the loss of SEB-reactive T cells (Fig. 2b). The *in vitro* responses to SEA (nonVβ8-TcR specific) were not affected in these mice. Multiple injections of Mls-1^{a+}-cells did not augment the deletion, nor did they induce a further reduction of the *in vitro* proliferative responses to

Figure 2. Intravenous immunization of superAg induces a state of anergy and causes a reduction of superAg-reactive T cell subpopulations. BALB/c mice were injected with Mls-1a cells or SEB at the indicated doses, and their lymph nodes harvested at day 14 post-Immunization for flow cytometry analysis and for *in vitro* reactivity to the relevant superAg. Shown in (A) are the Vβ6+ (—△—) and Vβ14+ (.▲.) T cell frequencies, and *in vitro* proliferative responses to irradiated Mls-1a splenocytes of DBA/2 origin (bar: □), and to irradiated allogeneic splenocytes of C3H/HeJ mice (bar: ▨), of lymph node cells of Mls-1a-injected mice. Shown in (B) are the Vβ8+ (—□—) and Vβ6+ (.■.) T cell frequencies, and *in vitro* proliferative responses to SEB (bar: □), and SEA (bar: ⁘), of SEB-injected mice. Results represent either Δ C.P.M. ± SEM, or T cell frequencies (%) ± SEM, among CD4+ T cells. Three to five mice were analyzed in each group.

Mls-1^{a}-immunized mice day 14



SEB-immunized mice day 14

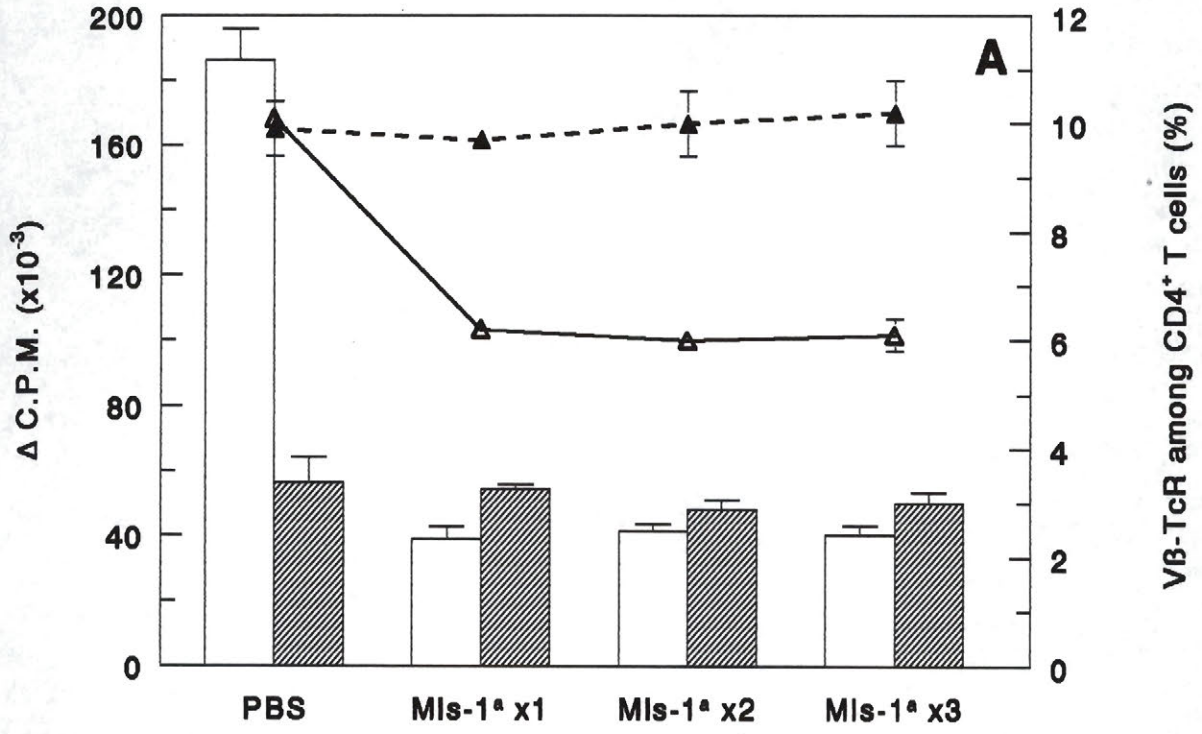


Mls-1^a (Fig. 3a). Thus, the latter observations show that the partial deletion of CD4$^+$/Vβ6$^+$ T cells cannot be enhanced by reexposing these cells to Mls-1^a molecules. The remnant of *in vitro* reactivity by Mls-1^a-anergic T cells could be attributable to Mls-1^a-reactive T cells which, despite the multiple injections of Mls-1^{a+}-cells, did not encounter the superAg *in vivo* (or at least not in a way that would normally induce anergy in these cells). Alternatively, the *in vitro* responses observed could be mounted against conventional antigens on Mls-1^a cells that are not "anergizing" due to either small antigen concentrations or to other reasons currently unknown to us. In contrast, repeated injections of SEB enhanced the deletion of CD4$^+$/Vβ8$^+$ T cells, reducing their relative frequencies from 13.4% (SEB injected 1 time) to 7.9% (SEB injected 5 times) (Fig. 3b). This enhanced reduction in reactive T cells was accompanied by a further slight decrease of *in vitro* reactivity to SEB. The discrepancies between Mls-1^a- and SEB-reactive T cell populations in their sensitivity to multiple injections may reflect a difference in the bioavailability of the two superAgs. Mls-1^a is an endogenous protein expressed by the injected cells, and is likely to persist in the periphery of the mice as long as the cells survive, whereas SEB is an exogenous protein, which makes it more susceptible to a rapid elimination. However, at the present time we can only speculate on the reasons for this difference.

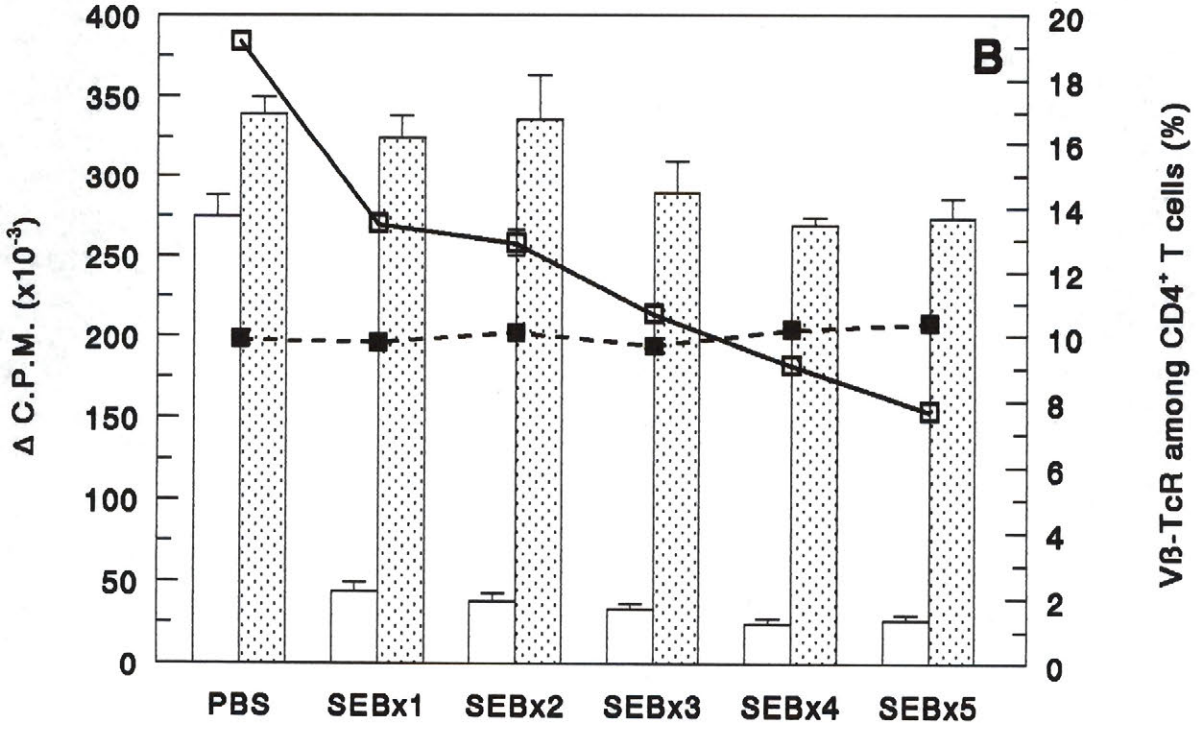
The superAg-induced anergy is not limited to a single mouse strain, since we and others (Rammensee, H.G., Kroschewski, R., and Frangoulis, B., 1989;

Figure 3. Effects of multiple injections of superAg on anergy and partial deletion of superAg-reactive T cell subpopulations. BALB/c mice were injected with 10^7 Mls-1a cells either once (x1) or every 4 days up to three times (x3), or with 50µg of SEB either once (x1) or every 4 days up to five times (x5). Lymph nodes were harvested 4 days after the last injection for flow cytometry analysis and for *in vitro* reactivity to the the relevant superAg. Shown in (A) are the Vß6+ (–△–) and Vß14+ (· ·▲· ·) T cell frequencies, and *in vitro* proliferative responses to irradiated Mls-1a splenocytes of DBA/2 origin (bar: □), and to irradiated allogeneic splenocytes of C3H/HeJ mice (bar: ▨), of lymph node cells of Mls-1a-injected mice. Shown in (B) are the Vß8+ (–□–) and Vß6+ (· ■ ·) T cell frequencies, and *in vitro* proliferative responses to SEB (bar: □), and SEA (bar: ⁘), of SEB-injected mice. Results represent either Δ C.P.M. ± SEM, or T cell frequencies (%) ± SEM, among CD4+ T cells. Three to five mice were analyzed in each group.

Mls-1^{a}-immunized mice



SEB-immunized mice



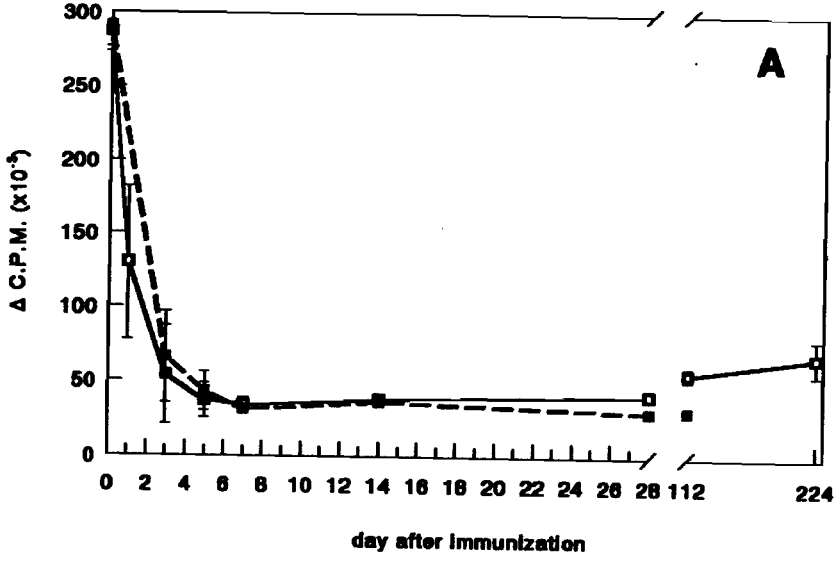
Rellahan, B.L., Jones, L.A., Kruisbeek, A.M., *et al.*, 1990; Webb, S., Morris, C., and Sprent, J., 1990) were successful at inducing anergy in various murine strains. However, in different strains the degree of peripheral deletion obtained can vary from none to about 75% in superAg-specific T cells.

In vivo kinetic studies of anergy and peripheral deletion induction in sham-thymectomized and thymectomized (Tx)-mice.

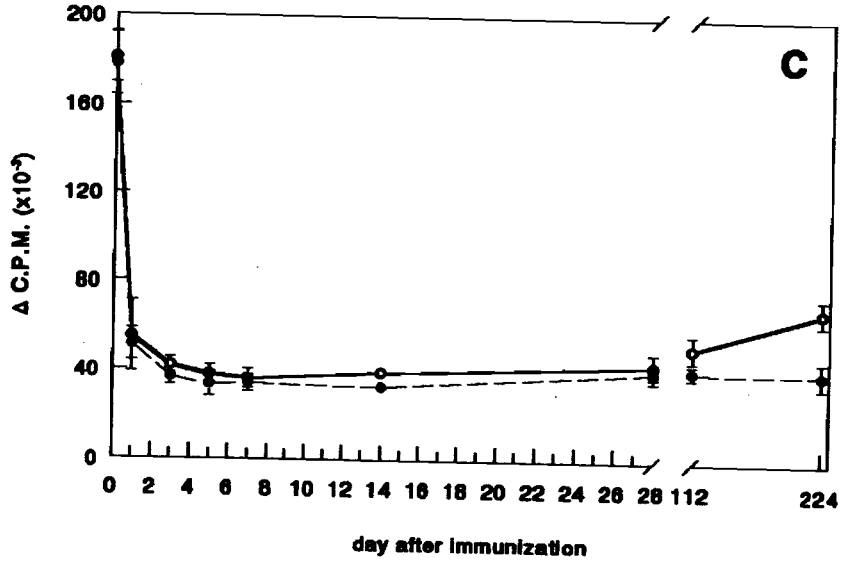
Injections of superAgs into Tx-mice enabled us to assess if anergy and deletion were peripheral phenomena. As shown in Fig. 4 (a and c), the induction of anergy in sham- and Tx-mice has the same kinetic profile, demonstrating that anergy induction does not require the presence of a thymus. *In vitro* proliferative assays showed that the low levels of reactivity to the superAg used for immunization are maintained for almost 100 days after immunization. Subsequently, superAg-reactivity increased slightly in superAg-treated sham-mice, possibly due to the generation of new superAg-specific T cells (see below). However, it should be noted that the level of reactivity observed by day 224 post-immunization in sham-mice remains low when compared to non-immunized mice (21% and 33% of normal proliferative responses for SEB and Mls-1^a respectively). We also observed, as have others (Kawabe, Y., and Ochi, A., 1990) that for SEB, the partial elimination of $CD4^+/V\beta8^+$ is preceded by an expansion of these cells on day 3 post-immunization, and is followed by a rapid decline that stabilized by day

Figure 4. ***In vivo*** **kinetics of anergy and deletion in superAg-treated sham or thymectomized (Tx) BALB/c mice. Mice were injected with either Mls-1a (10^7 cells) or SEB (100µg) , and killed at the indicated day after immunization for flow cytometry analysis, and for** ***in vitro*** **responses to the relevant superAg. SEB-injected sham mice (–□–) and SEB-injected Tx mice (· ·■· ·) were analyzed for** ***in vitro*** **reactivity to SEB (A), and for Vβ8+ T cell frequencies (B). Mls-1a-injected sham mice (–:–) and Mls-1a-injected Tx mice (· ·●· ·) were analized for** ***in vitro*** **reactivity to Mls-1a cells (C), and for Vβ6+ T cell frequencies (D). Results represent either Δ C.P.M. ± SEM (A and C), or T cell frequencies (%) ± SEM, among CD4+ T cells (B and D). Three to five mice were analyzed in each group.**

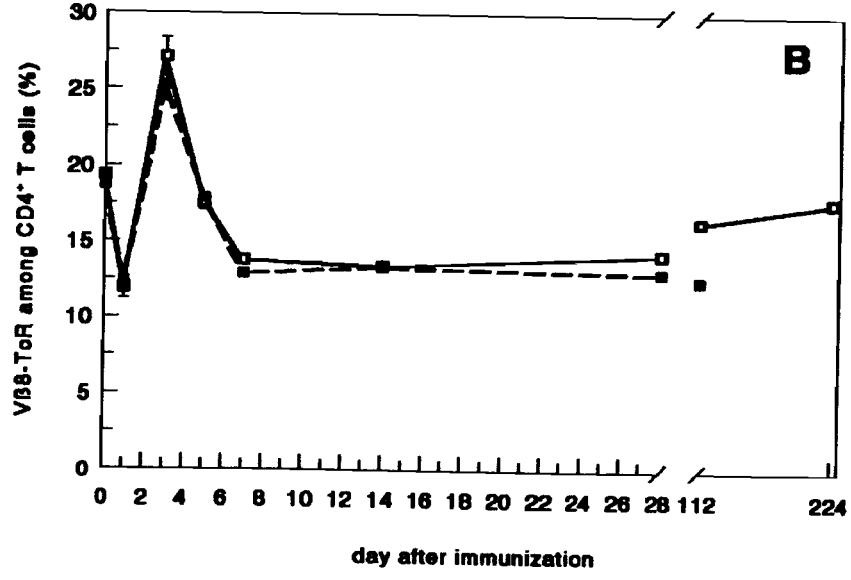
SEB-immunized mice



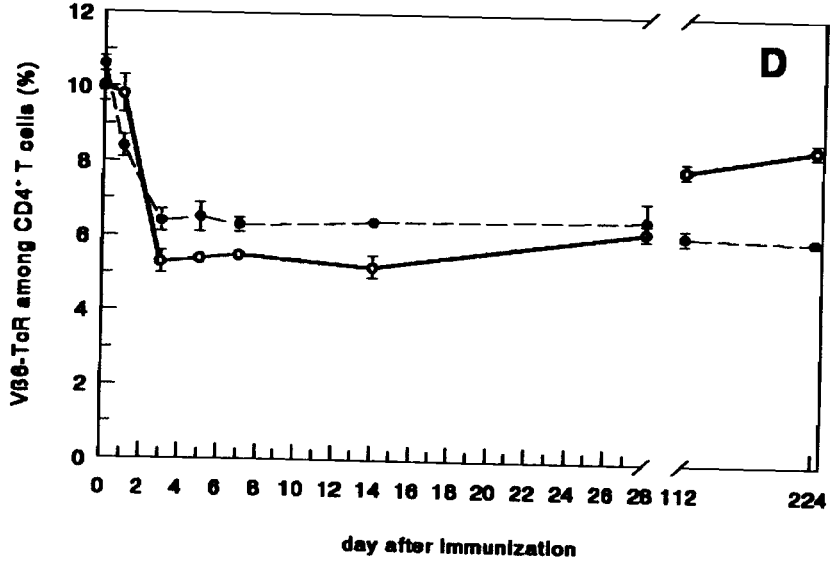
Mls-1ᵃ-immunized mice



SEB-immunized mice



Mls-1ᵃ-immunized mice



7 post-immunization (Fig. 4b). In Mls-1^a-injected BALB/c, no expansion was found in CD4$^+$/Vβ6$^+$ T cells prior to their deletion (Fig. 4d). The latter observation varies with different mouse strains. Webb and colleagues (Webb, S., Morris, C., and Sprent, J., 1990) found that, B10.BR (Mls-1^b, H-2^k) mice injected with AKR/J (Mls-1^a, H-2^k) splenocytes had a strong expansion of CD4$^+$/Vβ6$^+$ T cells in the first few days after immunization. This was subsequently followed by a deletion to about 25% of the initial levels by day 22 post-immunization. We also detected a $\approx$2% increase at day 3 post-immunization (from 14.2 $\pm$ 0.2 in PBS-injected vs 16.3 $\pm$ 0.1 in Mls-1^a-injected mice) before observing a deletion in CD4$^+$/Vβ6$^+$ T cells in CBA/CaJ (Mls-1^b/Mls-2^b, H-2^k) mice injected with CBA/J (Mls-1^a/Mls-2^a, H-2^k) splenocytes (unpublished data). However, as we report here for BALB/c (Mls-1^b, H-2^d) mice injected with DBA/2 (Mls-1^a, H-2^d) splenocytes, no expansion before deletion was observed in C3H/HeJ (Mls-1^b, H-2^k) mice injected with C3H.CE (Mls-1^a, H-2^k) splenocytes (Rammensee, H.G., Kroschewski, R., and Frangoulis, B., 1989). The reasons for these discrepancies are not known. In both SEB- and Mls-1^a-injected sham-mice a slow increase of superAg-specific T cell numbers is observed starting on day 30 post-immunization, which correspond to the increase of *in vitro* reactivity (Fig. 4). Since no increase in their reactivity to superAg was observed in these mice, this suggests that *de novo* T cells are responsible for the increase of *in vitro* superAg-reactivity.

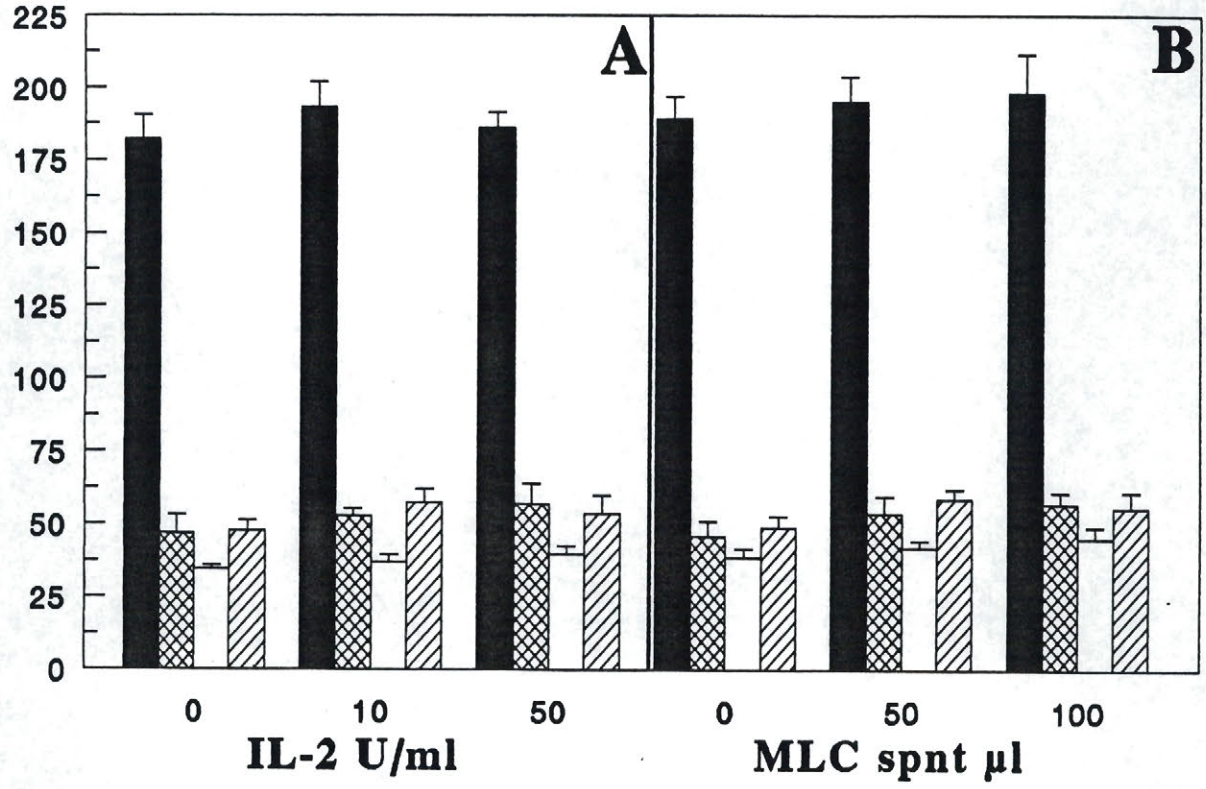
Effects of in vitro addition of T cell growth factors to anergic T cells.

Anergic T cells produce small amounts of IL-2 when stimulated with their specific superAg (Rammensee, H.G., Kroschewski, R., and Frangoulis, B., 1989; Rellahan, B.L., Jones, L.A., Kruisbeek, A.M., *et al.*, 1990; Kawabe, Y., and Ochi, A., 1990). To determine if the lack of proliferation of anergic T cells was caused simply by a lack of growth factors production, we added *in vitro* exogenous rIL-2 and MLC supernatant (Fig. 5). Addition of rIL-2 to Mls-1^a-anergized T cells (Fig. 5a) or to SEB-anergized T cells (Fig. 5c) had no significant effect on their proliferative responses to the superAg to which they were tolerant. Comparable results were obtained by the addition of MLC supernatant for both Mls-1^a- and SEB-anergized T cells (Fig. 5b and d respectively). Thus, anergic T cells fail to react to their specific superAg by at least two means: first, the blockage of IL-2 production, and second, the lack of responsiveness to T cell growth factors.

Anergic T cells are not suppressed by immunoregulatory T cells.

We wanted to insure that unresponsive T cells were not under the influence of antigen-specific suppressor T cells. Therefore, we added syngeneic Mls-1^a-anergized T cells to normal T cells of BALB/c origin (Mls-1^b, H-2^d), and challenged these cells *in vitro* with Mls-1^{a+} cells (DBA/2: Mls-1^a, H-2^d), or with Mls-1^b allogeneic spleen cells with disparate MHC (C3H/HeJ: Mls-1^b, H-2^k). In order to appreciate the reduction of the proliferative response due to the

Figure 5. ***In vitro*** addition of exogenous rIL-2 or MLC supernatant do not overcome superAg-induced anergy. BALB/c mice were injected with either 10^7 Mls-1a cells or SEB (50µg), and their lymph nodes were harvested at day 14 post-immunization for ***in vitro*** challenges with the relevant superAg. Exogenous IL-2 or MLC supernatant (spnt) were added or not at the begining of the ***in vitro*** proliferation assays. The proliferative responses of PBS- or Mls-1a-injected mice to irradiated Mls-1a splenocytes of DBA/2 mice, and to irradiated allogeneic splenocytes of C3H/HeJ mice, where rIL-2 (A) or MLC spnt (B) were added. PBS-injected with Mls-1a stimulator cells (■), and with allogeneic stimulator cells (▩); Mls-1a-injected with Mls-1a stimulator cells (□), and allogeneic stimulator cells (▨). The proliferative responses of PBS- or SEB-injected mice to SEB, and SEA , where rIL-2 (C) or MLC spnt (D) were added. PBS-injected stimulated with SEB (■), and with SEA (▩); SEB-injected stimulated with SEB (□), and with SEA (▨). Results represent Δ C.P.M. ± SEM.


A
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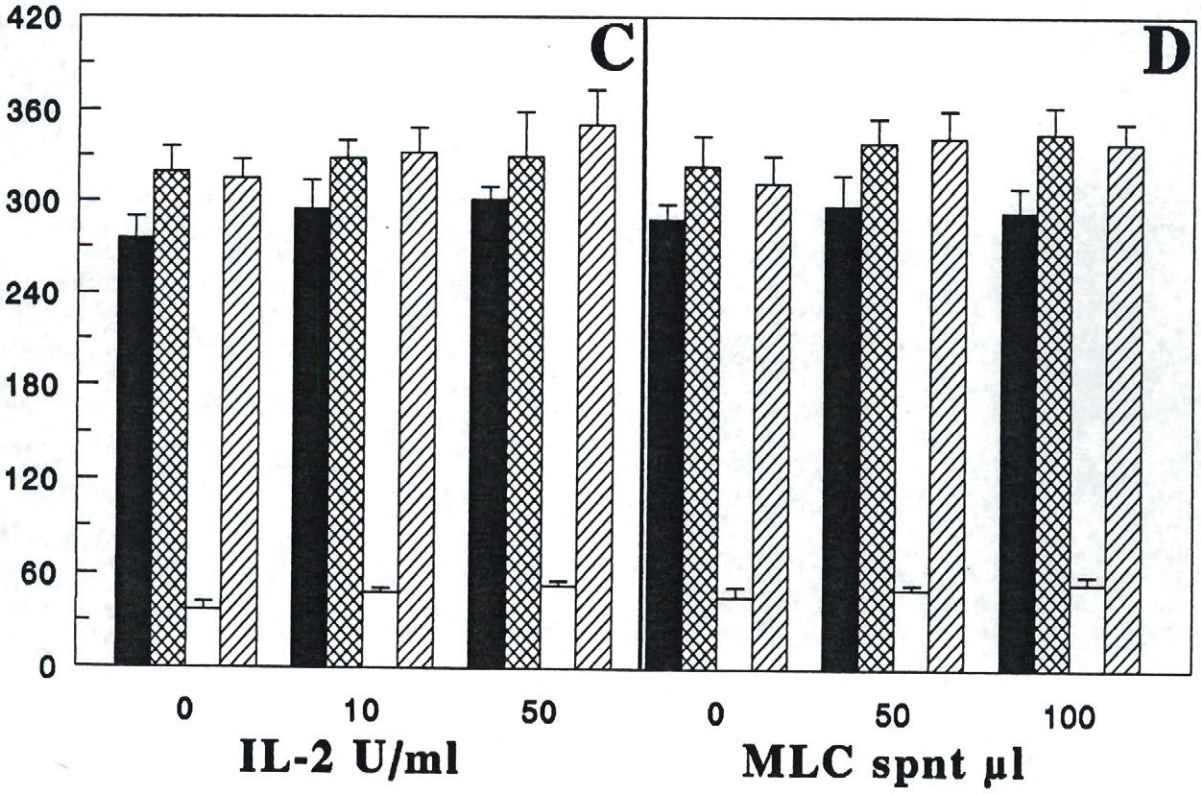
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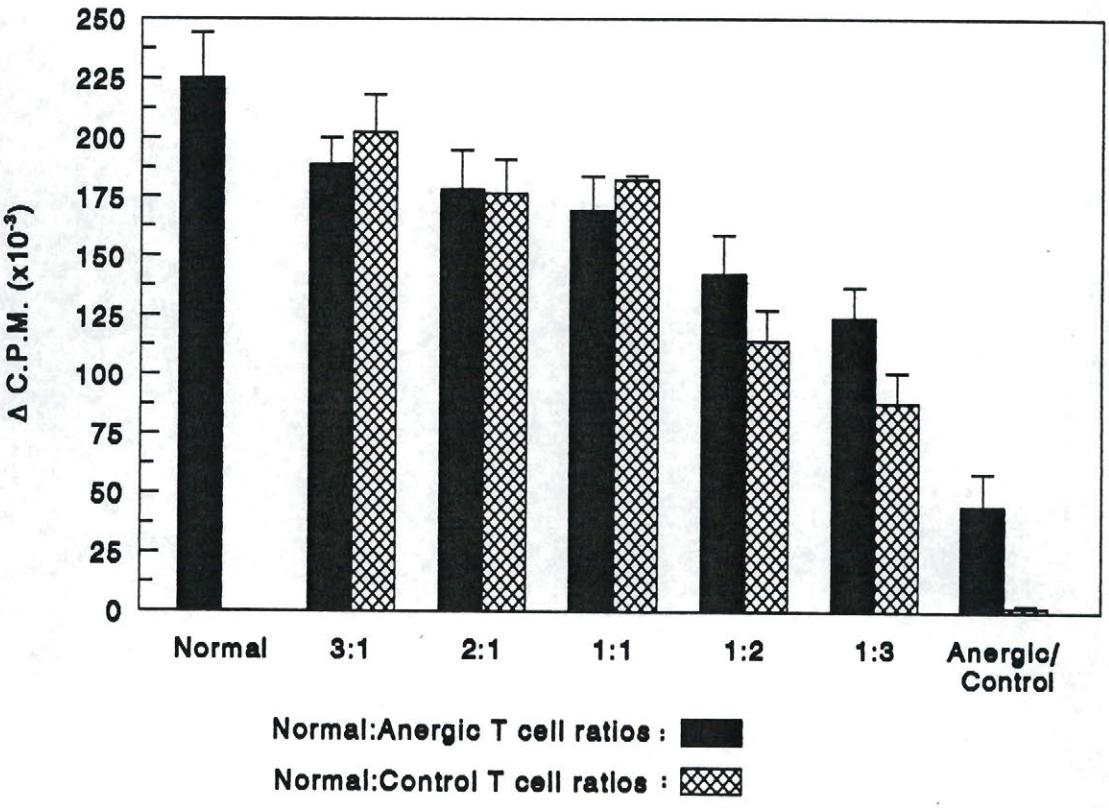
replacement of normal responder T cells by weakly-responding T cells (anergic T cells), we used as a control a mixture of normal responder T cells with T cells of DBA/2 (Mls-1^a, H-2^d) mice which do not respond to the syngeneic stimulating cells. DBA/2 mice possess only small numbers of potentially Mls-1^a-reactive T cells, and have the same MHC as the normal responder T cells (BALB/c: Mls-1^b, H-2^d), and therefore do not generate a significant MLC response to the BALB/c T cells (<3000 c.p.m.). In a typical antigen-specific suppression assays, a suppression of >80% can be observed with less than 10% of suppressive cells in culture (Adorini, L., Harvey, M.A., Miller, A. and Sercarz, E.E., 1979; Germain, R.N., and Benacerraf, B., 1981). As shown in Fig. 6, the proliferative responses of all the normal:anergic responder T cells mixtures were maintained at over 50% of the normal proliferative response. In samples with the higher concentration of anergic T cells (i.e., 75% of total T cells), the proliferative responses were on average at 55% of the normal proliferative response. Notably, the proliferative responses of the normal:anergic responder T cells ratios were comparable to those of the normal:control (DBA/2 T cells) mixtures. This argues strongly against a suppressive effect. The higher proliferation responses of the normal:anergic ratios of 1:2 and 1:3 over the normal:control of the same ratios are likely due to the contribution of non-anergized T cells of the anergic population to the normal response. The allogeneic responses did not vary significantly in all groups and ratios studied (data not shown). Based on these results, it seems

unlikely that the superAg model of anergy induction is caused by immunoregulatory T cells.

Figure 6. Weak *in vitro* proliferative response of anergic T cells is not caused by immunoregulatory T cell suppression. BALB/c mice were injected with either 10^7 Mls-1a cells or PBS, and their lymph nodes were recovered at day 14 post-immunization for *in vitro* challenges with Mls-1a. Purified-T cells from PBS-treated mice (Normal) were mixed at the indicated ratios with purified-T cells of Mls-1a-injected mice (Anergic), or with purified-T cells of lymph nodes taken from Mls-1a+ DBA/2 mice (Control). Normal:Anergic responder T cells were stimulated with either irradiated Mls-1a splenocytes of DBA/2 mice (■), or with irradiated allogeneic splenocytes of C3H/HeJ mice (not shown); Normal:Control responder T cells were stimulated with irradiated Mls-1a splenocytes of DBA/2 mice (▩) to evaluate the dilution factor caused by anergic T cells in the aforementioned proliferative assays. Results represent Δ C.P.M. ± SEM.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225
200
175
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Δ C.P.M. (x10⁻³)
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3:1
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Control
Normal:Anergic T cell ratios :
Normal:Control T cell ratios :

4. Discussion

SuperAgs stimulate large numbers of mature T cells bearing specific Vβ-segments of the TcR. They achieve this by binding to both TcR Vβ-segments and MHC class II molecules of APCs. This property has proven extremely useful for studying immunological phenomena, especially T cell tolerance (reviewed in Herman A., Kappler, J.W., Marrack, P. and Pullen, A., 1991). SuperAgs provided the means for obtaining conclusive evidence for thymic clonal deletion of autoreactive T cells and enabled the demonstration of peripheral clonal anergy and deletion (Kappler, J.W., Staerz, U.D., White, J., and Marrack, P.C., 1988; Rammensee, H.G., Kroschewski, R., and Frangoulis, B., 1989; Rellahan, B.L., Jones, L.A., Kruisbeek, A.M., *et al.*, 1990; Kawabe, Y., and Ochi, A., 1990). In this study we investigated the peripheral forms of tolerance induced by superAgs. We investigated the effects of dose and injection regimen, *in vivo* kinetics, effects of T cell growth factors on anergic T cells, including the possible contribution of specific suppression mechanisms for maintaining the anergic condition.

We found that augmenting the immunizing dose (in mice given only one dose) had no effect on either anergy or deletion. This was true even for Mls-1^a-injected mice that received numbers of donor cells 1000 times larger than the numbers required to reach the maximum degree of anergy and deletion. Unfortunately, due

to its toxic effects, SEB could not be injected at doses that exceeded 100μg. Nevertheless, a dose of 100μg of SEB was still 4 times higher than the minimal dose needed for optimal anergy and deletion induction. Although multiple superAg injections had no effect on peripheral anergy and deletion in Mls-1^a-injected mice, in SEB-injected mice increasing numbers of injections produced a concomitant decrease of SEB-reactive T cell levels and *in vitro* proliferative responses to SEB. The reason for the discrepancies between Mls-1^a and SEB is unknown, but several factors could be evoked like different rate of SuperAg elimination, different tissue distribution, or different types of APC.

SuperAgs injections in Tx-mice revealed that the superAg-induced anergy and partial deletion were peripheral events. Tx- and sham-mice had similar kinetic profiles for both anergy and deletion. Long term studies in these mice showed that anergy lasted at least 112 days for SEB-immunized Tx-mice and for more than 224 days in Mls-1^a-immunized Tx-mice. *In vitro* reactivity to the superAg used for immunization in sham-mice increased concomitantly with superAg-reactive T cell numbers. However, even at day 224 post-immunization the proliferative responses were still low when compared to PBS-injected mice, 21% and 33% of normal responses for SEB- and Mls-1^a-injected sham mice respectively. These findings show that anergy is a long lasting phenomenon. Following the initial phase of T cell deletion, there is no evidence of further loss of anergic T cells. It should be noted that these cells are also non-responsive to direct cross-linking of their TcR

with anti-Vβ-TcR MoAbs in the presence of splenocytes, even if exogenous rIL-2 is added (our unpublished data and Rellahan, B.L., Jones, L.A., Kruisbeek, A.M., 1990). Thus it seems unlikely that anergic cells respond to other antigen once "anergized".

The unresponsive state of anergic T cells is not simply due to a limitation of IL-2 production by these cells (Rammensee, H.G., Kroschewski, R., and Frangoulis, B., 1989; Rellahan, B.L., Jones, L.A., Kruisbeek, A.M., *et al.*, 1990; Kawabe, Y., and Ochi, A., 1990). We and others (Rammensee, H.G., Kroschewski, R., and Frangoulis, B., 1989; Kawabe, Y., and Ochi, A., 1990) have found that the addition of rIL-2 directly to culture wells could not reverse the unresponsive state of anergic T cells. MLC supernatant also had no effect on the proliferative responses of these cells. The inability of anergic T cells to respond to exogenous IL-2 could be due to the lack of high-affinity IL-2 receptor expression or to an impaired signal transduction induced by these receptors. However, since the IL-2 receptors were shown to be expressed on anergic T cells of both Mls-1^a- and SEB-immunized mice (Rammensee, H.G., Kroschewski, R., and Frangoulis, B., 1989; Rellahan, B.L., Jones, L.A., Kruisbeek, A.M., *et al.*, 1990), it would appear that the IL-2 responsiveness defect is more likely associated with signal transduction events.

A role for suppressor T cells was suggested by some authors, to explain the lack of responsiveness in anergic cells. Using a model where lethally irradiated

(DBA/2xB10.D2)F1 mice grafted with B10.D2 cells developed severe graft-versus-host reaction (GVHR) to non-H-2 antigen (Halle-Pannenko, O., Pritchard, L.L., Motta, R., and Mathé, G., 1978), Bruley-Rosset and colleagues (Bruley-Rosset, M., Miconnet, I., Canon, C., and Halle-Pannenko, O., 1990) observed that the mortality rate could be markedly reduced by donor pre-immunization against Mls-1^{a+} cells. This effect was attributed to Mls-1^{a} generated double-negative suppressor T cells. In order to determine if suppressor T cells were active in our models, we mixed purified T cell of PBS-injected control mice with purified T cells of Mls-1^{a}-immunized mice in various proportions and challenged them with Mls-1^{a+} cells. The results showed no evidence of suppression in these cell mixing experiments. It was important to confirm the validity of the superAg-induced tolerance as an anergy model since it is the only alternative to the T cell anergy observed in TcR transgenic mice. Moreover, the superAg-induced anergy model has the particular advantage of providing an internal control in the populations of T cells bearing non-reactive TcR-Vβ segments.

Despite the increasing attention devoted to superAgs in recent years, we remain unsure as to how they induce T cell anergy in mice, especially in view of their potent T cell mitogenic properties. SuperAgs are presented by MHC class II molecules without antigen processing by APCs (Mollick, J.A., Cook, R.G., and Rich, R.R., 1989; Fraser, J.D., 1989). This enables these molecules to stimulate T cells directly by simultaneously binding the TcR and class II molecules

(Fleischer, B., Schrezenmeier, and Conradt, P., 1989). Thus, T cells can be triggered by any class II-expressing cells, including some that may not be competent APCs, with the consequence that the T cell may not receive the proper costimulatory signals required for complete activation. Such lack of costimulatory signals in $CD4^+/T_H1$ clones has been reported to induce an anergic state in these cells (reviewed in Mueller, D.L., Jenkins, M.K., Schwartz, R.H., 1989). The costimulatory signal needed for full activation of these clones was found to be transmitted by the T cell's CD28 receptor after ligation with the BB-1/B7 molecule expressed on the surface of competent APCs (Jenkins, M.K., Taylor, P.S., Norton, S.D., Urdhal, K.B., 1991; Linsley, P.S., Brady, W., Grosmaire, L., Aruffo, A., *et al.*, 1991). Since the weak expression of the BB-1/B7 molecule has been associated with the poor stimulatory property of resting B cells (Jenkins, M.K., Taylor, P.S., Norton, S.D., Urdhal, K.B., 1991), it is conceivable that the superAg-induction of anergy is caused by resting B cell presentation of superAg to reactive-T cells.

Our study demonstrates that following *i.v.* injection of superAgs anergy and partial deletion are thymus-independent events, and that the state of anergy is long-lasting and cannot be reversed by addition of T cell growth factors. We also ruled out the contribution of suppressor cells in the maintenance of anergy. Thus, we believe that the superAgs provide acceptable tools for the study of peripheral tolerance phenomena.

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CHAPTER III.

CYCLOSPORIN A MARKEDLY ENHANCES SUPERANTIGEN-INDUCED PERIPHERAL T CELL DELETION AND INHIBITS ANERGY INDUCTION

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1. Summary

Cyclosporin A (CsA) is a well known immunosuppressive agent that modulates immune tolerance in many ways. CsA can give rise to a state of long-term non-immunosuppressed transplantation tolerance, but it can also aggravate autoimmune diseases, and provoke specific forms of autoimmunity. These effects, which are often paradoxical, remain largely unexplained. In this study, we investigated the effects of CsA on superantigen (superAg)-reactive peripheral T cells. The intravenous injection of either staphylococcal-enterotoxin B (SEB), or Mls-1^a cells into Mls-1^b recipients, causes long-term *in vitro* non-responsiveness (anergy) and partial elimination of the peripheral T-cell-receptor (TcR)-Vβ8$^+$/CD4$^+$ and -Vβ6$^+$/CD4$^+$ T cell subsets respectively. We report that CsA markedly enhances the peripheral elimination of SEB- and Mls-1^a-reactive T cells such that up to 90% of the targeted CD4$^+$/Vβ-subpopulations are deleted. The degree of deletion depends on the dose and the schedule of CsA administration, and the number of superAg injections. In situations where the extent of deletion is only moderate, we find that the remaining superAg-reactive T cells fail to develop anergy, unlike the T cells of control superAg-immunized mice. Higher doses of CsA are required to enhance T cell deletion ($\geq$25 mg/kg/d *i.p.*) than to impair anergy induction ($\geq$ 6.25 mg/kg/d *i.p.*). In view of these results, it appears that the degree of tolerance in CsA/superAg-treated mice depends on the balance between these

opposing effects, i.e., enhancement of peripheral elimination versus the abrogation of anergy. The possibility of enhancing or preventing immune tolerance with a drug may have important clinical implications.

2. Introduction

CsA is a potent immunosuppressive agent used clinically to control allograft rejection, graft-versus-host-disease (GVHD), and some autoimmune diseases (1). It is well accepted that the principal inhibitory action of CsA is the result of impaired lymphokine production by T cells, especially IL-2 (2-4). In rats, a form of long-term non-immunosuppressed tolerance to allografts can be induced by a short course of CsA treatment (5-6). The underlying mechanism is not well understood, but may depend on the generation of antigen-specific suppressor cells (5,7). Paradoxically, in some circumstances, CsA treatment can have detrimental effects which include the induction of autoimmune diseases and syngeneic-GVHD (reviewed in ref.8). It has been suggested that CsA induces syngeneic GVHD by blocking intrathymic clonal deletion (9-10). However, our results (11) and those of Bryson *et al* (12) do not support this interpretation. Alternatively, CsA could affect tolerance by acting on peripheral T cells. In this study, we analyzed the effects of CsA therapy on the peripheral tolerance induced by superantigens (superAg). Two superAgs were used, staphyloccocal-enterotoxin B (SEB) and Mls-1^a (a product of the intrinsic mouse mammary tumor virus 7 (MMTV-7)) (13), which stimulate T-cell-receptor (TcR) V$\beta 8^+$ and TcR-V$\beta 6^+$/CD4^+ T cells respectively (14-15). These superAgs are known to cause both a partial elimination and a long-term *in vitro* anergy in their targeted T cell subpopulations (16-20). We

found that superAg recognition during the course of CsA treatment leads to a considerable enhancement of the peripheral elimination of the reactive T cells. This elimination process is superAg specific. With protocols that provoke an extensive elimination of superAg-reactive T cells, the proliferative responses to these antigens *in vitro* are very low. In contrast, CsA abrogates the development of anergy which usually occurs following injection of these superAgs. The latter effect is only apparent when sufficient numbers of undeleted T cells remain after CsA/superAg-treatment. Relatively low doses of CsA could inhibit anergy induction, while higher doses were required to enhance T cell deletion. The blockage of anergy by CsA may be of importance in experimental models where CsA induces autoimmune phenomena. Based on our results, we postulate that CsA can induce a state of tolerance by provoking antigen-specific T cell elimination, but can sometimes paradoxically increase responses by preventing anergy induction.

3. Materials and Methods

Mice

BALB/c mice (H-2^d, Mls-1^b, Mls-2^a, Lyt-1.2, Lyt-2.2), DBA/2 mice (H-2^d, Mls-1^a, Mls-2^a, Lyt-1.1, Lyt-2.1) were purchased from Charles River Laboratory (St-Constant, Quebec, Canada), and C3H/HeJ mice (H-2^k, Mls-1^b, Mls-2^a) from the Jackson Laboratory (Bar Harbor, ME). Adult BALB/c mice (4 to 6 weeks old) were thymectomized in our facility as described (21) and rested for 7 days before the onset of treatments. All the experiments were performed in female mice.

Monoclonal antibodies (MoAb's) and Reagents

Phycoerythrin(PE)-conjugated anti-CD4, PE-conjugated anti-CD8, anti-Lyt-1.1, anti-Lyt-2.1, and anti-Thy-1.2 were purchased from Cedarlane (Hornby, Canada). The following MoAb's were produced as culture supernatants and purified by protein G (Pharmacia, Montreal, Quebec, Canada) affinity chromatography: anti-Vβ6 (44-22-1 hybridoma, ref. 22), anti-Vβ8.1-8.2 (KJ16 hybridoma, ref. 23), anti-Vβ8.1-8.2-8.3 (F23.1 hybridoma, ref. 24), anti-Vβ14 (14.2 hybridoma, ref. 25), and anti-TcR-$\alpha\beta$ (H57-579 hybridoma, ref. 26). For flow cytometry analysis the purified MoAbs were directly conjugated to fluorescein isothiocyanate (FITC). CsA was a kind gift of Sandoz (Dorval, Canada). SEA was purchased from Toxin Technology (Sarasota, FL) and SEB from was purchased from Sigma Chemical

Co. (St-Louis, MO).

SuperAg-immunization and CsA treatments

SuperAg immunizations were performed by tail vein injections of either 10^7 Mls-1^{a+} splenocytes (RBC depleted), or 50-100μg of SEB. Cells or SEB were injected in a volume of 0.25ml of PBS. The day of superAg injection was considered day 0 of our experiments. CsA was suspended in olive oil (OO) (20 mg/ml) and given intraperitoneally at a dose of 50mg/kg/day, unless otherwise indicated. CsA was administered daily starting one day before superAg immunization (day -1) and continued until the day the mice were killed, unless otherwise indicated. Control groups included untreated mice, CsA-treated mice (no superAg), superAg-immunized mice (no CsA), and superAg- and diluent (OO)-treated mice. In control mice, CsA and/or diluent were administered in the same dose and/or volume as in the experimental groups.

Flow cytometry analysis

For two color flow cytometry analysis, lymph nodes (LN) cells (pooled cervical, axillary, para-aortic and mesenteric LN) were stained with either PE-anti-CD4 or PE-anti-CD8 and with one of the FITC-labelled-anti-Vβ MoAbs mentioned above. One color flow cytometry analysis was performed with FITC-labelled MoAbs only. Briefly, $5x10^5$ LN cells were incubated with PE-conjugated MoAb

for 15 minutes at 4°C, then washed 3 times with PBS containing 1% FCS (Gibco, Burlington, Canada) and then incubated in the same conditions with the second FITC-labelled-anti-Vβ MoAb. Propidium iodide (Sigma Chemical Co.) was added after the last wash. Dead cells were excluded based on propidium iodide staining and forward scatter. In each sample, 10^4 cells were analyzed on a FACScan (Becton and Dickenson, Missisauga, Canada) flow cytometer, and results were plotted on a logarithmic scale. In some experiments spleen cells were analyzed. Before staining, spleens cells (RBC depleted) were depleted of B cells by two rounds of sequential J11.D2 MoAb and complement treatment, performed as we have previously described (27). Following depletion of dead cells on a lympholyte-M gradient (Cedarlane), spleen cells were stained and analyzed as described above for LN cells.

In vitro proliferative assays

LN cells were cultured in 96 flat bottom plates (Gibco) in quadruplates at $5x10^5$ cells/well with either: a) for SEB stimulation, with 10^6 syngeneic irradiated (2000 rads) splenocytes of untreated BALB/c mice and 10μg/ml of SEB, or 1μg/ml of SEA as a control; or b) for Mls-1^a experiments, with 10^6 irradiated (1000 rads) DBA/2 splenocytes or irradiated (1000 rads) allogeneic C3H/HeJ (H-2^k, Mls-1^b) splenocytes. Cells were cultured in RPMI-1640 (Gibco) containing 10% FCS, L-glutamine, $5x10^{-5}$M 2-mercaptoethanol and antibiotics. Cells were pulsed with

[^{3}H]-thymidine (ICN, Montreal, Canada) at day 3 of culture and harvested on day 4, unless otherwise indicated. Days in culture refer to the day that the cells were harvested. In all cases, the cells were harvested after 18 hour incubation with [^{3}H]-thymidine. In the case of stimulation with either SEA or SEB, ▲cpm = (cpm experimental) - (cpm control [no superAg]). In the case of Mls-1^a or MLC stimulation, ▲cpm = (cpm experimental) - (cpm with syngeneic stimulator cells).

4. Results

Enhanced elimination of superAg-reactive T cells in CsA/superAg-treated mice

When injected to an appropriate recipient, Mls-1^{a+} cells and SEB induce a partial elimination of the specific superAg-reactive T cells (19-20, and our results in Figures 1 and 2). We find that the elimination of superAg-reactive T cells is markedly augmented by CsA administration. As shown in Table 1 and Figure 1 (a to d), the frequency of $CD4^+/V\beta6^+$ T cells (reactive to Mls-1^a) in Mls-1^b recipients treated with CsA (50 mg/kg/d) decreases to less than 20% of the original values in BALB/c mice. The $CD4^+/V\beta8.1^+$ T cells, another Mls-1^a-reactive subset, may also be affected by CsA/Mls-1^a-treatment, since there was a partial decrease in $KJ16^+$ ($V\beta8.1^+/V\beta8.2^+$)/$CD4^+$ T cell population (data not shown). The loss of $CD4^+/V\beta6^+$ T cells in CsA/Mls-1^a-treated mice represents a 3.6 fold greater deletion, when compared to the control Mls-1^a-immunized (non-CsA treated) groups (Table 1). The deletion is specific since the frequency of $CD4^+/V\beta14^+$ T cell (which do not react to Mls-1^a antigen) remained relatively unchanged. The enhanced deletion of $CD4^+/V\beta6^+$ T cells can be observed in both the spleen and lymph nodes of treated mice (Table 1). Generally, the CsA/Mls-1^a-induced elimination is completed by day 7 post-immunization (data not shown). Comparable results were obtained in CsA-treated CBA/CaJ (Mls-1^b) mice injected with CBA/J (Mls-1^a) spleen cells (data not shown).

Table 1. *CsA Enhances Elimination of Mls-1^a-reactive CD4$^+$/V$_\beta$6$^+$ T Cells in Mls-1^a-treated Mice*

Mls-1^a donor	Mls-1^b recipient	CsA treatment	V$_\beta$6 among CD4$^+$ day 0‡	day 3	day 14	day 28	V$_\beta$6 among CD8 day 0‡	day 28
DBA/2	BALB/c	–	9.9 ± 0.5 (10.3 ± 0.7)	5.2 ± 0.6	5.3 ± 0.3 (7.1 ± 0.8)	6.2 ± 0.8	11.9 ± 1.2	11.3 ± 1.3
		+	10.2 ± 0.5 (10.4 ± 0.2)	4.3 ± 0.2	1.7 ± 0.1 (2.4 ± 0.9)	1.7 ± 0.2	11.0 ± 0.9	10.7 ± 1.4
		+§ (interrupted)			3.3 ± 0.2			
			V$_\beta$14 among CD4 day 0‡	day 3	day 14	day 28	V$_\beta$14 among CD8 day 0‡	day 28
DBA/2	BALB/c	–	9.4 ± 0.2 (9.8 ± 0.1)	9.5 ± 0.3	10.3 ± 0.4 (9.9 ± 0.2)	9.9 ± 0.7	4.0 ± 0.3	4.5 ± 0.4
		+	8.9 ± 0.5 (9.9 ± 0.0)	9.9 ± 0.2	10.4 ± 0.2 (10.9 ± 0.1)	10.0 ± 0.5	4.0 ± 0.5	4.8 ± 0.2
		+§ (interrupted)			10.7 ± 0.0			

* Day 0 represents the day of superAg immunization. Mice received either no CsA (–) or a daily injection of 50 mg/kg of CsA beginning on day –1, until either day 3, 14, or 28 as indicated. LN cells were analyzed by two-color flow cytometry (see Fig. 1). Values in parentheses represent analysis of B cell–depleted spleen cells.

‡ These mice were not injected with Mls-1^a cells. T cell frequencies for CsA-treated control mice are expressed as the mean for all CsA-treated control groups (i.e., mice were injected with CsA from day –1 to either day 3, 14, or 28). V$_\beta$6$^+$ and V$_\beta$14$^+$ T cell frequencies were closely similar in these three groups.

§ CsA was administered on day –1 and days 2–14 (i.e., a 2-d interruption of CsA treatment).

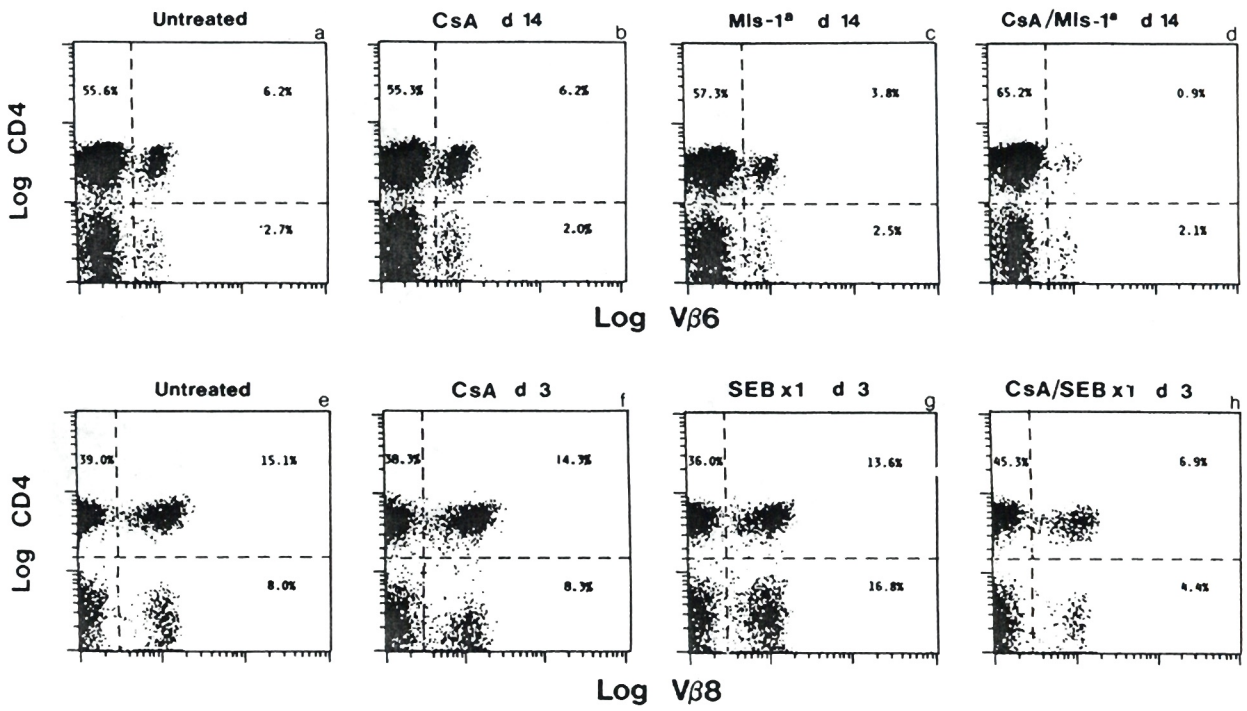

Figure 1. Enhanced elimination of superAg-reactive T cells in CsA/superAg-treated mice. Day 0 refers to the day of superAg (Mls-1ª or SEB) injection. CsA (50 mg/kg/d) was administered from day −1 of the experiments until either day 3 or 14 as indicated. Histograms show two-color flow cytometry analysis of BALB/c LN cells of untreated mice (*a* and *e*); CsA-treated control mice (*b* and *f*); Mls-1ª-immunized mice (*c*); CsA-treated and Mls-1ª-immunized (CsA/Mls-1ª) mice (*d*); SEB-immunized mice (*g*); and CsA-treated SEB-immunized (CsA/SEB) mice (*h*). Staining was performed with CD4-PE and either anti-Vβ6-FITC (44-22-1 mAb) or anti-Vβ8-FITC (F23.1, anti-Vβ8.1, 8.2, 8.3 mAb). 10,000 cells were analyzed in each histogram and results are plotted on a logarithmic scale.

A CsA enhanced T cell elimination process was also observed in SEB-immunized mice. A single injection of SEB after the initiation of CsA therapy (50 mg/kg/d) caused a rapid decline in the numbers of the reactive $CD4^+/V\beta8^+$ T cells, as demonstrated in Figure 1 (e to h) and Table 2, unlike in the SEB-immunized control animals. This early decline was apparent at day 3 (Table 2), and was observed within 36 hours post-SEB-immunization (data not shown). However, with only one SEB injection, CsA did not greatly enhance the deletion observed at day 12 and 30 (Table 2). When a second injection of SEB (SEBx2) was performed at day 7, the long-term deletion of $CD4^+/V\beta8^+$ T cells became clearly more pronounced in CsA-treated than in non-CsA-treated mice (Table 2). Multiple additional injections of SEB in CsA-treated mice greatly enhanced the elimination of $CD4^+/V\beta8^+$ T cells, such that their frequency decreased to 1.8% (Figure 2). This represents less than 10% of the frequency of $CD4^+/V\beta8^+$ T cell normally found in BALB/c mice. In mice that received a single injection of SEB, CsA provoked the loss of approximately half of the $CD8^+/V\beta8^+$ T cells, while there was only a small reduction in the numbers of these cells in control mice (Table 2). Unlike $CD4^+/V\beta8^+$ T cells, the elimination of $CD8^+/V\beta8^+$ T cells was not further augmented by multiple injections of SEB (Table 2 and data not shown). The CsA/SEB elimination process is superAg specific, as shown by the unaltered (or slightly increased) frequency of $CD4^+/V\beta6^+$ and $CD8^+/V\beta6^+$ T cells (Table 2).

Table 2. *CsA Enhances Elimination of $V_\beta 8^+$ T Cells in SEB-treated Mice*

BALB/c SEB injected	CsA treatment	SEB-reactive $V_\beta 8$ subset*					
		$V_\beta 8$ among CD4				$V_\beta 8$ among CD8	
		day 0‡	day 3	day 12	day 30	day 0‡	day 30
x1	–	23.7 ± 0.5	24.8 ± 0.9	13.2 ± 0.3	13.4 ± 0.5	41.6 ± 1.6	35.0 ± 1.4
	+	24.2 ± 0.9	11.9 ± 0.8	11.6 ± 0.6	8.9 ± 0.9	40.1 ± 2.0	16.3 ± 2.5
	+ (stopped day 20)§	22.9 ± 1.1			8.5 ± 0.3		15.9 ± 1.8
x2	–			12.4 ± 0.2	12.7 ± 0.3		18.3 ± 0.3
	+			6.5 ± 0.3	5.2 ± 0.2		14.2 ± 0.6
		Control $V_\beta 6$ subset					
		$V_\beta 6$ among CD4				$V_\beta 6$ among CD8	
		day 0‡	day 3	day 12	day 30	day 0‡	day 30
x1	–	9.9 ± 0.6	9.5 ± 0.4	12.2 ± 0.2	11.9 ± 1.1	11.5 ± 1.1	13.1 ± 0.7
	+	10.2 ± 0.2	13.0 ± 1.2	13.8 ± 0.6	11.5 ± 0.3	11.6 ± 0.6	14.9 ± 0.8
	+ (stopped day 20)§	9.8 ± 0.4			10.5 ± 0.6		15.0 ± 0.2
x2	–			12.6 ± 0.1	12.1 ± 0.9		14.4 ± 1.1
	+			13.7 ± 1.0	14.3 ± 1.0		15.2 ± 2.1

* Day 0 represents the day of the first super Ag immunization. Mice received one (x1) or two (x2) injections of 100 μg of SEB. The second injection of SEB was performed at day 7. Mice received either no CsA (–) or 50 mg/kg of CsA daily from day –1, until either day 3, 12, or 30 as indicated. LN cells were analyzed by two-color flow cytometry (see Fig. 1). $V_\beta 8^+$ T cells were detected with the F23.1 mAb (anti-$V_\beta 8.1$, 8.2, 8.3).

‡ These mice were not injected with SEB. T cell frequencies for CsA-treated control (non-SEB-injected) mice represent the mean of the three groups injected with CsA from day –1 to either day 3, 12, or 30. $V_\beta 8^+$ and $V_\beta 6^+$ T cell frequencies were closely similar in these three groups.

§ These mice received CsA from day –1 to day 20.

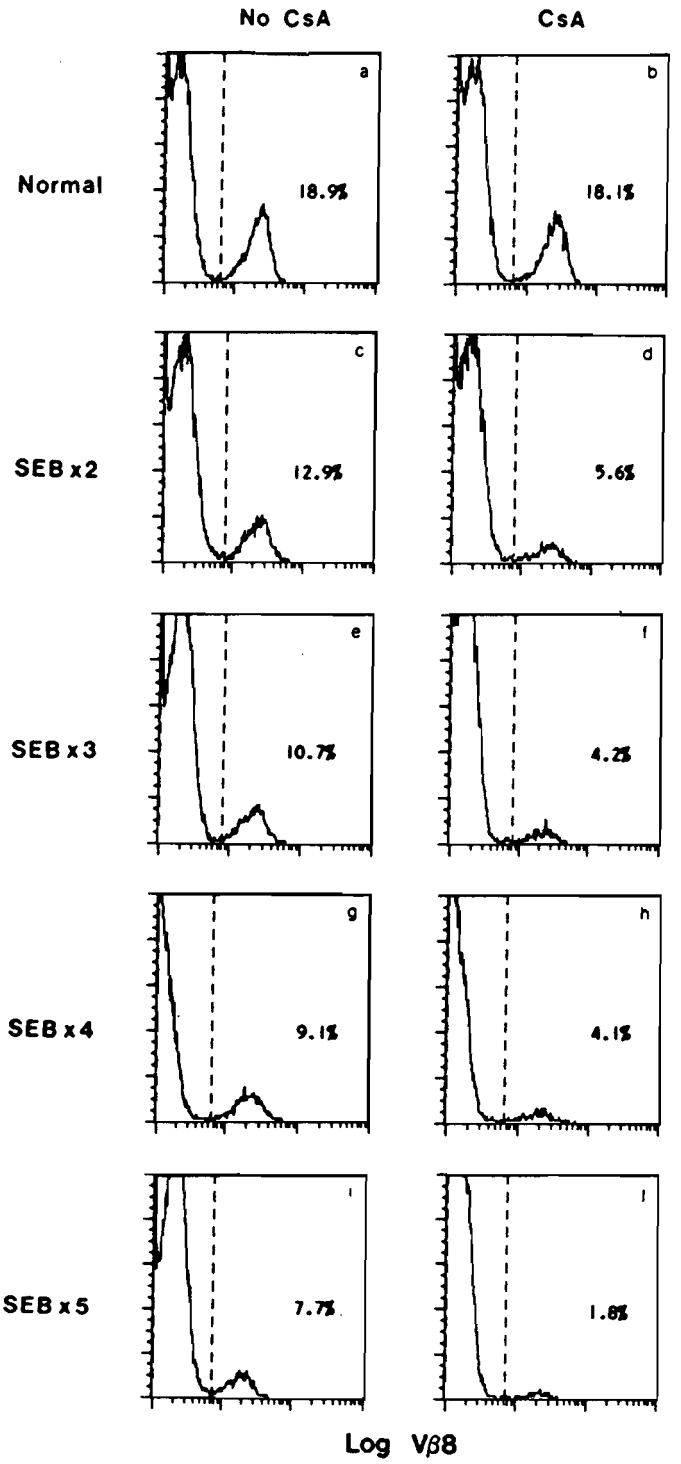

Figure 2. Marked enhancement of $CD4^+/V_\beta8^+$ T cell deletion with multiple injections of SEB in CsA-treated mice. Single-color histograms represent the relative numbers of gated LN $CD4^+$ cells (y-axis) stained with FITC-conjugated anti-$V_\beta8.1$–8.2 (KJ16) mAb (x-axis). SEB (50 μg) was injected intravenously either once (SEBx1) or every 4 d up to five times (SEBx5) and the mice were killed 4 d after the last injection. The histograms on the left are from non-CsA-treated mice, while those on the right are from CsA-treated mice. CsA treatment (50 mg/kg/d) was begun 1 d before the first SEB injection and continued until the day at which the mice were killed. Representative results are shown.

Effect of CsA dose on T cell deletion

SuperAg-induced T cell deletion was enhanced in mice injected with CsA at doses of 25 mg/kg/d, and even more so at doses of 50 mg/kg/d, but not in mice injected with lower CsA doses or diluent (OO) (Figure 3). While CsA enhanced T cell deletion, it inhibited the development of anergy that was observed in control mice (see below under in vitro stimulation assays).

CsA/superAg-induced elimination in thymectomized (Tx) mice and the effects of CsA-treatment termination

To determine if the T cell deletion was a peripheral event, adult BALB/c mice were thymectomized (Tx) 7 days prior to CsA-treatment and/or Mls-1^a-immunization. As shown in Table 3, the extent of elimination of $CD4^+/V\beta6^+$ T cells in CsA/Mls-1^a-treated Tx mice was equivalent to that observed in the euthymic groups.

When CsA treatment was stopped for 8 days in CsA/Mls-1^a-treated Tx mice (Table 3) or 10 days in euthymic CsA/SEB-treated mice (Table 2), we observed no change in superAg-reactive T cell frequency. Thus withdrawal of CsA does not result in a rapid increase of superAg-reactive T cell numbers.

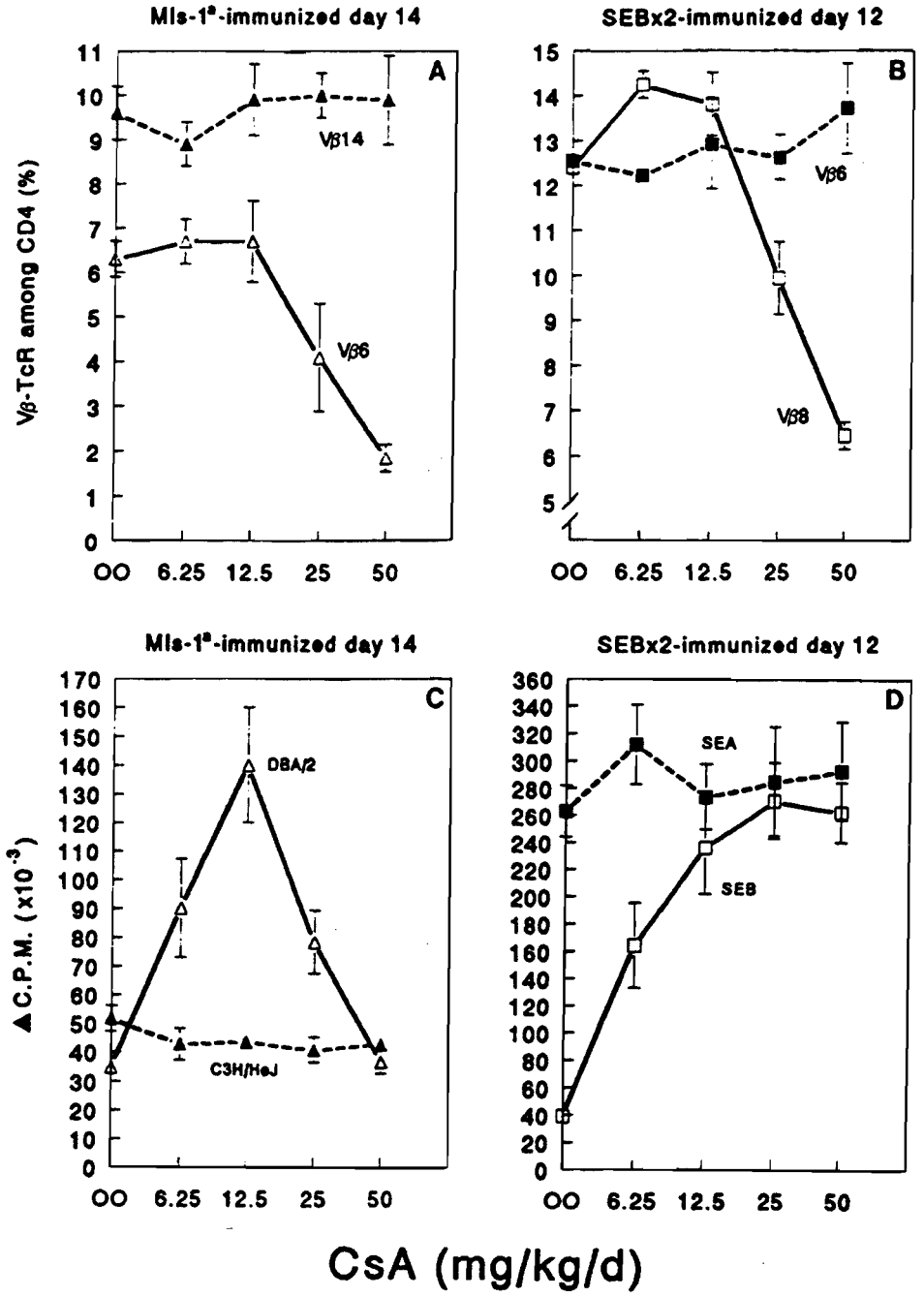

Figure 3. Effects of CsA dose on LN T cell numbers and in vitro responses to superAgs. BALB/c mice were injected with either Mls-1ª cells or SEBx2. Immunizations and analyses were performed as described in Tables 1 and 2. Mice were treated with OO, or various doses of CsA, from day −1 to either day 12 (SEB-immunized mice) or to day 14 (Mls-1ª-injected mice). (*A*) $V\beta6^+$ (△) and $V\beta14^+$ (▲) T cell numbers in Mls-1ª-immunized mice; (*B*) $V\beta8^+$ (□) and $V\beta6^+$ (■) T cell numbers in SEB-injected mice; (*C*) proliferative responses of LN cells to either DBA/2(H-2^d, Mls-1ª) (△) or C3H/HeJ (H-2^k, Mls-1^b) (▲) irradiated stimulator cells, in Mls-1ª-immunized mice; (*D*) proliferative responses of LN cells to either SEB (□) or SEA (■), in SEB-immunized mice. Results represent either percent T cell numbers ± SEM, among $CD4^+$ cells (*A* and *B*), or Δ cpm ± SEM (*C* and *D*). Three to five mice were analyzed in each group.

Table 3. *Enhanced $CD4^+/V_\beta6^+$ T Cell Elimination by CsA Treatment in the LN of Thymectomized Mls-1^a-immunized BALB/c*

CsA treatment*	$V_\beta6$ among $CD4^+$ cells			
	day 0‡	day 3	day 14	day 28
–	10.6 ± 0.2	6.4 ± 0.3	6.5 ± 0.0	6.5 ± 0.5
Continuous	10.3 ± 0.4	6.4 ± 0.1	2.2 ± 0.1	2.4 ± 0.2
Stopped at day 20§	10.2 ± 0.2	–	–	2.2 ± 0.4

* Mice received either no CsA (–) or 50 mg/kg/d of CsA from day –1, up until either day 3, 14, or 28 as indicated.
‡ Mice not immunized with Mls-1^a cells.
§ These mice received CsA from day –1 to day 20.

T cell marker analysis of lymph nodes and spleen cells

The reduction of superAg-reactive T cells that we observed could have been secondary to either deletion or to downregulation of the TcR. In addition, in the Mls-1^a-injected groups (i.e., Mls-1^a alone or CsA/Mls-1^a-treated mice) there might have been an expansion of donor T cells, which would reduce the relative frequency of the host Vβ-subpopulations. To answer these questions, we analyzed the expression of several T cell markers in these mice. We found that the lymph nodes of CsA/Mls-1^a-treated mice had closely similar frequencies of cells expressing either Thy-1, TcR-$\alpha\beta$, or CD4 + CD8 (Table 4). Thus, it seems unlikely that there were significant numbers of T cells bearing conventional T cell markers but negative for the TcR due to downregulation. Moreover, in both Mls-1^a- and SEB-treated groups, no variation in TcR intensity was detected in the superAg-reactive T cell populations (Figures 1 and 2).

In Mls-1^a-treated BALB/c mice the analysis of Lyt-1 and Lyt-2 alleles in the spleen (depleted of B cells by J11D.2 and complement treatment) or the lymph nodes revealed that virtually all T lymphocytes were of host origin (Table 4). Consequently, non-Vβ6 expressing donor T cells could not have been replacing or diluting host T cells.

Table 4. *T Cell Marker Analysis of LN and Spleen Cells in CsA/Mls-1^a-treated BALB/c Mice at Day 14*

Group*	$V_\beta6^+$/CD4$^+$‡	$V_\beta14^+$/CD4$^+$‡	TCR-α/β^+§	CD4$^+$/CD8$^+$‡	Thy-1.2$^+$‡	Lyt-1.1$^+$§	Lyt-2.1$^+$§
Untreated	6.0 ± 0.1	5.2 ± 0.3	74.4 ± 0.2	73.6 ± 0.3	75.2 ± 0.7	0.0 ± 0.1	0.0 ± 0.0
						(0.0 ± 0.0)	(0.0 ± 0.0)
CsA	5.9 ± 0.2	4.8 ± 0.1	73.7 ± 0.4	71.8 ± 0.8	72.9 ± 1.0	0.0 ± 0.0	0.0 ± 0.0
						(0.2 ± 0.2)	(0.2 ± 0.1)
Mls-1^a	3.4 ± 0.3	5.6 ± 0.6	75.3 ± 0.5	75.8 ± 0.1	74.7 ± 0.9	0.0 ± 0.1	0.0 ± 0.0
						(0.0 ± 0.0)	(0.1 ± 0.1)
CsA/Mls-1^a	0.7 ± 0.2	5.2 ± 0.3	67.9 ± 0.4	66.4 ± 0.5	65.8 ± 1.2	0.0 ± 0.3	0.1 ± 0.3
						(0.0 ± 0.0)	(0.1 ± 0.2)

* The groups are the same at those described in Fig. 1.
‡ $V_\beta6^+$/CD4$^+$ cells and $V_\beta14^+$/CD4$^+$ cells were enumerated by two-color flow cytometry with CD4-PE and either anti Vβ6-FITC or anti-Vβ14 FITC. TCR-α/β^+ and Thy-1.2$^+$ cells were analyzed by one-color staining. CD4$^+$/CD8$^+$ represents the sum of CD4$^+$ and CD8$^+$ T cells analyzed in two colors. Positive cells are expressed as a percent of total LN cell numbers ± SEM.
§ DBA/2 (donor) cells are Lyt-1.1$^+$ and Lyt-2.1$^+$. BALB/c (recipient) cells are Lyt-1.2$^+$ and Lyt-2.2$^+$. In DBA/2 spleens a mean of 27.5% of cells were Lyt-1.1$^+$, and a mean of 13.5% of cells were Lyt-2.1$^+$ (data not shown). Values in parentheses represent cell numbers in B cell–depleted BALB/c (recipient) spleen cells.

In vitro stimulation assays

Intravenous injection of Mls-1^{a+} cells or SEB results in a state of anergy (proliferative non-responsiveness) when T cells are challenged with the same antigen *in vitro* (16-18). As shown in Figure 4a, the T cells of mice injected with SEB once (SEBx1) or five times (SEBx5) are strongly anergic, while responses to SEA are unchanged. The development of anergy was not observed when CsA (50 mg/kg/d) was given in SEBx1-immunized mice (Figure 4a). The proliferative capacity to SEB remained comparable to untreated mice (Figure 4a). However, the elimination of SEB-reactive T cells was much more extensive in CsA/SEBx5-treated mice (SEB injected 5 times), and in that case the SEB stimulated proliferation was drastically reduced (Figure 4a). Presumably, the latter low responses were due to the extensive T cell elimination.

The inhibition of anergy induction was apparent at CsA doses as low as 6.25 mg/kg/d, although higher doses were more effective (Figure 3). It is noteworthy that mice injected with 6.25 mg/kg/d or 12.5 mg/kg/d of CsA did not have enhanced T cell deletion (Figure 3). In mice injected with 50 mg/kg/d of CsA, the blockage of anergy induction after Mls-1^{a} treatment was difficult to assess, because these mice had low numbers of CD4^{+}/Vß6^{+} cells (Table 1, Figure 3). The low proliferative responses observed with Mls-1^{a} stimulation (Figure 4b) could be secondary to the deletion event as well as to delayed onset of anergy. However, when high dose CsA treatment was temporarily interrupted, e.g., at days 0 and 1,

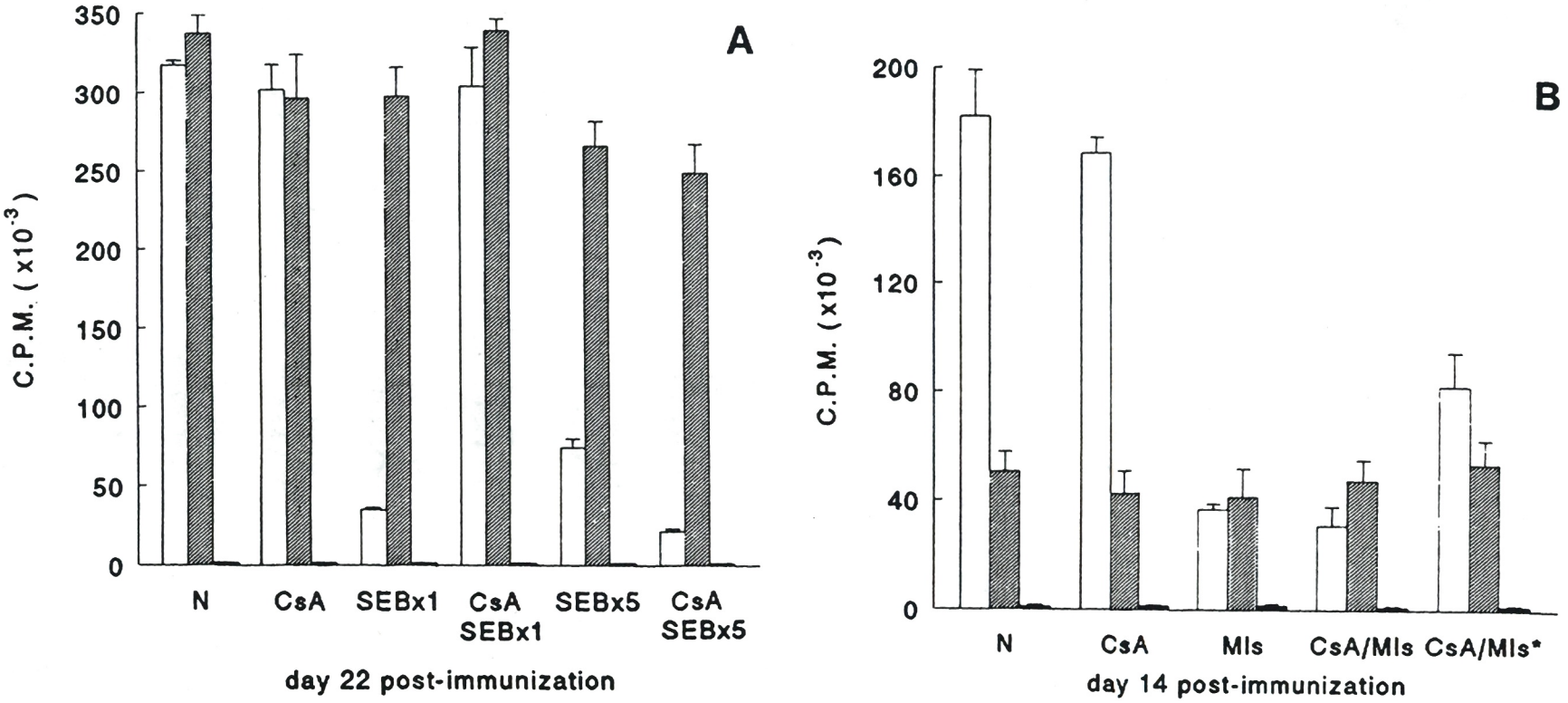

Figure 4. Inhibition of SuperAg-induced clonal anergy by CsA. (*A*) The proliferative responses of the LN cells of BALB/c mice with SEB (*open bars*) or SEA (*hatched bars*) in vitro stimulation (cells harvested at day 4 of culture). Mice were treated with CsA and SEB as explained in the legend to Fig. 2. Results represent the mean cpm ± SEM (four to five mice in each group). (*B*) The in vitro proliferative responses of the LN cells of BALB/c mice treated with CsA and Mls-1^a cell injections as explained in the legend to Fig. 1. LN cells were stimulated either with irradiated DBA/2 (H-2^d, Mls-1^a) spleen cells (*open bars*) or irradiated C3H/HeJ (H-2^k, Mls-1^b) spleen cells (*hatched bars*). The results respresent the mean cpm (cells harvested at day 4 of culture) ± SEM (5–15 mice in each group). Stimulation with syngeneic cells resulted in less than 1,700 cpm in all cases (*filled bars*).

mice had an intermediate extent of deletion of $CD4^+/V\beta6^+$ T cells (Table 1) and a stronger *in vitro* proliferative responses to Mls-1^a stimulation (Figure 4b). It appears that in the latter case CsA had prevented anergy, while causing insufficient deletion to abrogate the proliferative responses.

Webb *et al* (20) have reported that early after Mls-1^a-immunization, the $CD4^+/V\beta6^+$ T cells had a strong proliferative response to Mls-1^a, but the peak response was seen at day 3 of *in vitro* cultures rather than day 4 for normal (untreated) mice. However, this form of accelerated kinetics is not observed at day 14 post-immunization (Figure 5b, and ref. 20), at which time the maximal proliferative response is weak. Accordingly, in CsA and non-CsA treated groups, we find that the maximal proliferative responses at day 14 post-Mls-1^a-immunization and at day 22 post-SEB-immunization occur as with usual proliferative kinetics (day 4 peak) (Figure 5). SuperAg-immunized mice (non-CsA treated) had low peak responses despite the presence of substantial numbers of potentially Mls-1^a reactive ($CD4^+/V\beta6^+$) and SEB reactive ($CD4^+/V\beta8^+$) T cells, and these T cells can be markedly reduced in numbers by CsA treatment (Table 1 and 2).

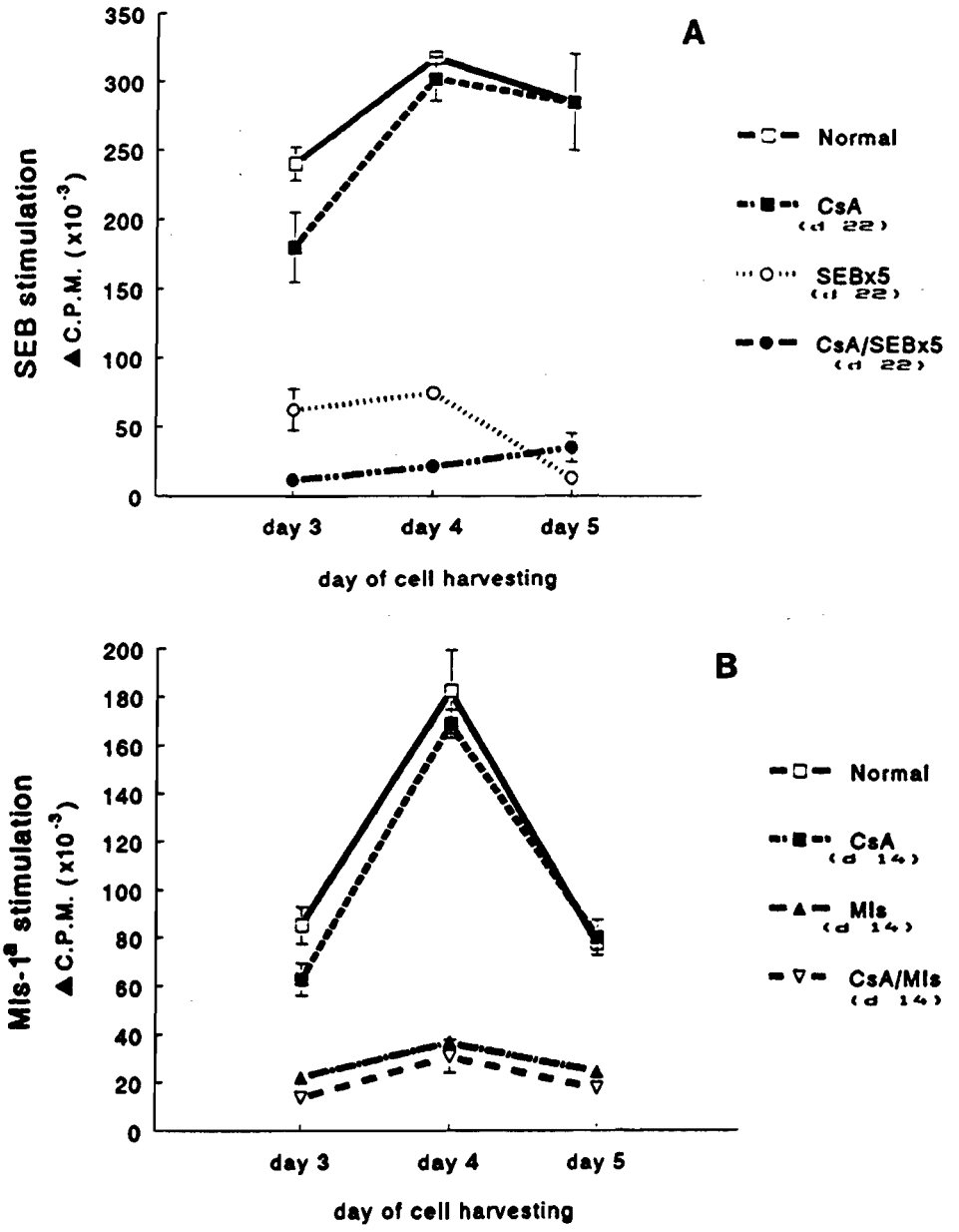

Figure 5. Kinetics of the peak proliferative responses in mice immunized with superAg and/or CsA treated. The groups of mice presented here are the same as in Fig. 4. (*A*) LN cells of BALB/c mice were stimulated with SEB in vitro. The results represent the mean Δcpm ± SEM. (*B*) LN cells of BALB/c (H-2^d, Mls-1^b) mice (treated as indicated) were stimulated with DBA/2 (H-2^d, Mls-1^a) irradiated spleen cells. The results represent the mean Δcpm ± SEM. In both panels the peak proliferative response was observed at day 4 of culture in all groups studied, except in the CSA/SEBx5 group, where responses were maximal at day 5 (the latter responses were very low). Stimulation with syngeneic stimulator cells (no superAg) yielded less than 1,500 cpm in all cases.

5. Discussion

This study was prompted by the numerous reports showing that CsA can alter immunologic tolerance (1, 8). These effects are poorly understood and often contradictory, since in some cases a short course of CsA treatment has induced tolerance to allografts, while in other experimental models this drug has provoked autoimmune phenomena (8). Enhanced tolerance has often been attributed to the action of suppressor cells (5,7), while the paradoxical induction of autoimmune diseases has been imputed to defective thymocytic differentiation (9-10). Nevertheless, for a variety of reasons, we hypothesized that CsA could also act by affecting peripheral T cell tolerance mechanisms (i.e., peripheral deletion and anergy).

Our study shows that CsA treatment can markedly enhance the deletion of superAg-reactive T cells in either Mls-1^a-immunized or SEB-immunized mice. Without CsA treatment, the partial T cell elimination observed in superAg-immunized mice is limited and preceded by an expansion of the targeted T cells (Refs. 19-20, and our unpublished observations). Not surprisingly, CsA prevents this expansion phase. Nonetheless, the CsA/superAg-induced deletion occurs earlier and is more extensive than with superAg immunization alone. Under optimal conditions, the extent of T cell elimination in CsA/superAg-treated mice can vary from 3 to 4 fold greater than in non-CsA-treated superAg-immunized

mice. In mice receiving CsA, up to 90% of reactive $CD4^+$ T cells can be eliminated. Thus, with both superAgs, CsA treatment results in an earlier and more extensive deletion process, seen at the time when clonal expansion is often apparent in control superAg-immunized mice.

A combined CsA and superAg treatment had no effect on non-reactive T cell Vβ-subpopulations, other than a slight increase in their frequencies which likely resulted from the loss of the targeted subsets. Thus, CsA appears to enhance the deletion of only those T cells that are responding to superAgs. No sign of downmodulation of the TcR was detected, and the cessation of CsA administration did not result in a rapid increase in the frequency of reactive T cells. In addition, in the Mls-1^a-injected groups, (i.e., Mls-1^a alone or CsA/Mls-1^a) we found no detectable levels of donor T cells in the lymph nodes or the spleen. This excludes the possibility of a massive expansion of non-V$\beta6^+$ donor T cells that could reduce the relative frequency of the host T cell Vβ^+-subpopulations. In view of these results, we believe that the disappearance of superAg-reactive T cells in CsA/superAg-treated mice results primarily from a deletion event rather than an alternative process.

The deletion observed in thymectomized (Tx) mice treated with CsA and Mls-1^{a+} cells was similar to that of euthymic mice. Therefore, the CsA/superAg-induced T cell deletion is a peripheral event, as reported by others in non-CsA-treated superAg-immunized mice (19-20).

The T cells of superAg-immunized mice usually display a markedly reduced *in vitro* proliferative response to the same superAg (16-18). This condition is generally referred to as anergy. Recently, some authors have questioned the anergic status of Vβ6$^+$ T cells in Mls-1^a-immunized mice (20). They showed that early after immunization with Mls-1^{a+} cells, the low responses to Mls-1^a were due to the different kinetics of responses in these mice (i.e., previously activated T cells had an earlier peak proliferative response). At day 14 post-immunization they observed (as we do) low proliferative responses to Mls-1^{a+} cells with no accelerated kinetics effect. They attributed the loss of strong *in vitro* proliferative responses to the partial elimination of superAg-reactive T cells. However, our results do not support this conclusion, since there were substantial numbers of undeleted CD4$^+$/Vβ6$^+$ T cells at day 14 post-Mls-1^a-immunization (without CsA), that could be markedly reduced by CsA treatment. Therefore, we conclude that most of the unresponsive CD4$^+$/Vβ6$^+$ T cells of Mls-1^a-immunized mice (non-CsA-treated) must be potentially Mls-1^a reactive since their deletion is specifically provoked by CsA, and that these T cells are anergic.

Similarly, our results provide strong evidence that most of the nonresponsive Vß8$^+$ T cells of SEB-immunized mice are in fact TcR-SEB specific, and can be specifically deleted with CsA treatment. The superAg specificity of the majority of unresponsive Vß6$^+$ T cells (Mls-1^a immunized mice) and unresponsive Vß8$^+$ T cells (SEB immunized mice) has not been previously demonstrated. It is

noteworthy that even with maximal CsA-enhanced deletion there was still some residual (but low) proliferation to either Mls-1^a or SEB. Thus, not all superAg-reactive cells were deleted. In addition, it is not unlikely that some of the residual (undeleted) T cells are not superAg specific. However, if this is the case, such nonspecific cells would represent less than 10% of all CD4$^+$/Vß6$^+$ and CD4$^+$/Vß8$^+$ T cells, based on the percent of cells that resist deletion.

CsA clearly prevents the development of anergy in SEB-treated mice. However, this is only apparent in mice that have moderate deletion of CD4$^+$/Vβ8$^+$ T cells, as in mice injected with SEB only once. In mice with severe deletion of CD4$^+$/Vβ8$^+$ T cells, e.g., mice injected 5 times with SEB, the *in vitro* proliferative responses to this superAg are very low. This indicates that extensive deletion of reactive T cells can counteract the strong *in vitro* proliferative responses due to the blockage of anergy by CsA. This is also true in the case of Mls-1^a-immunized mice. Our results show that lower doses of CsA are required to prevent anergy induction than to enhance T cell deletion. This explains why mice injected with low doses of CsA had consistently higher in vitro proliferative responses to superAg than control mice (superAg immunized).

The mechanism(s) by which CsA can prevent the induction of anergy is not clear. CsA is known to block a Ca^{2+}-mediated T cell activation pathway (28-30). Interestingly, Jenkins *et al* (31-32) have shown that the induction of anergy in CD4$^+$/Th1-type T cell clones depends on a Ca^{2+}-mediated signal. CsA could

prevent the induction of anergy in these clones (33). This raises the possibility that CsA may inhibit anergy induction *in vivo* by altering a Ca^{2+}-dependant signal. However, the mechanisms of anergy induction in superAg-immunized mice are poorly understood, and our study did not address how CsA acts in this process.

The work of Shi *et al* (34) demonstrates that CsA blocks programmed-cell-death (apoptosis) in the thymus. However, CsA does not prevent radiation-induced apoptosis in mature T cells (35). Nevertheless, it was surprising to find that CsA could enhance peripheral T cell deletion. One possibility is that peripheral T cells are not eliminated by apoptosis, but the recent studies of Kawabe and Ochi (19) suggest that SEB may induce peripheral T cell apoptosis. Interestingly, we found that the stimulation of T cells *in vitro* with matrix-bound anti-Vβ8 antibodies in the presence of CsA, induces a complete and specific elimination of these cells, apparently by apoptosis (L.E. Vanier and G.J. Prud'homme [chapter IV]). These preliminary data suggest that CsA may enhance apoptosis in mature antigen-reactive T cells.

We can only speculate on the mechanism by which CsA enhances peripheral deletion, but Duke and Cohen (36) have shown that IL-2 deprivation of activated T cells results in death by apoptosis. Since CsA blocks IL-2 production (2-4) and interferes with anergy induction (as presented in this article), the T cells activated by superAgs may be left with no source of IL-2. This could possibly result in apoptosis and loss of reactive T cells in CsA/superAg-treated mice.

CsA/antigen-induced elimination of T cells may represent a mechanism of tolerance to allografts. CsA has been shown to induce long-term non-immunosuppressed tolerance to allografts in many species (37). This occurs, for example, in rats pre-treated with CsA and donor specific blood transfusion before allograft transplantation (6). Tolerance to allografts has usually been attributed to antigen-specific suppressor cells (5,7). However, in accordance with our findings with superAg, a recent study with limiting dilution analysis suggests that CsA may induce tolerance to allografts by provoking the elimination of graft-reactive T cells (38). On the other hand, since CsA blocks anergy it is possible that if the conditions of treatment used do not favour an extensive T cell deletion, then CsA may actually enhance immune responses and cause adverse effects. In fact, CsA was shown to aggravate some autoimmune diseases, and to sometimes induce specific forms of autoimmunity (reviewed in 8). Cyclosporin-induced syngeneic (or autologous) GVHD, in particular, has been extensively studied (39-43). This condition occurs after withdrawal of CsA, and is clearly an autoimmune disease that can be adoptively transferred with T cells. Some investigators have suggested that this disease is due to a blockage of intrathymic clonal deletion in CsA-treated mice (9-10), resulting in the production of autoaggressive T cells. However, we (11) and others (12) did not detect elevated numbers of peripheral forbidden (superAg-reactive) T cells in mice with CsA-induced syngeneic GVHD. In fact, based on our current study it is possible that even if such T cell clones were not

deleted in the thymus, they would be at least partially deleted in the periphery under the influence of CsA. However, as shown by our results, CsA does not necessarily induce a complete deletion of superAg-reactive T cells. For example, CsA-treated mice receiving only one injection of SEB had a moderate deletion of $CD4^+/V\beta8^+$ T cells, and had enhanced *in vitro* responses to SEB compared to non-CsA-treated controls. Several SEB injections were required to induce a maximal T cell deletion. Similarly, low doses of CsA blocked anergy induction, but did not enhance clonal deletion. Conceivably, since CsA blocks anergy, this drug could induce autoimmune states or aggravate autoimmune diseases in cases where there is only a low or moderate deletion of autoaggressive T cells and a blockage of anergy induction. In CsA-induced syngeneic GVHD, the effector T cells may consist of T cells that are normally tolerized primarily by anergy rather than intrathymic deletion. This hypothesis is consistent with the observation that in some mouse strains CsA treatment induces this disease, despite apparently normal intrathymic clonal deletion (12).

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CHAPTER IV.

EFFECTS OF CYCLOSPORIN A, RAPAMYCIN AND FK-520 ON PERIPHERAL T CELL DELETION AND ANERGY

by

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1. Abstract

We investigated the effects of cyclosporin A (CsA), FK520 (FK, a close analogue of FK506 with similar immunosuppressive properties), and rapamycin (RAPA) on peripheral T cell deletion and anergy. In a previous study (1 [chapter III]) we showed that CsA could enhance superantigen-induced T cell deletion, but inhibited anergy in residual T cells. In this study, we show that FK and RAPA, unlike CsA, have no effects on peripheral T cell deletion and anergy. CsA-enhanced deletion is not unique to superantigen-stimulated responses, since this drug can also markedly enhance $CD4^+$ (but not $CD8^+$) T cell deletion in mice treated with an anti-TcR$\alpha\beta$ MoAb. Under the influence of CsA, residual T cells show prolonged downmodulation of the TcR. The maintenance of severe T cell deletion requires continuous daily CsA treatment, and TcR downmodulation appears to depend on the presence of circulating MoAb, since it gradually reverses unless MoAb is re-administered. Moreover, the TcR is rapidly re-expressed in culture. *In vivo*, anti-TcR$\alpha\beta$ MoAb treatment does not induce T cell anergy (irrespective of the presence of CsA). *In vitro*, CsA enhances apoptosis in anti-TcR MoAb or staphylococcal enterotoxin B (SEB)-stimulated T cells. T cell elimination is greatest in highly purified T cell populations, and is inhibited by the addition of APCs to the cultures. CsA-enhanced T cell elimination is not mediated by phagocytic cells and occurs without prior proliferation. The absence of effects

of FK on deletion and anergy suggests that CsA exerts its effects through a calcineurin-independent pathway(s). The different effects of CsA, FK and RAPA on peripheral T cells may be relevant to the development of tolerance in some models.

2. Introduction

We have previously shown that cyclosporin A (CsA) enhances the deletion of T cells stimulated *in vivo* with superantigens (superAg) (1 [chapter III]). Deletion was only observed in the superAg-reactive T cell populations, and under optimal conditions resulted in the loss of up to 90% of responding cells. On the other hand, CsA prevented the induction of anergy in the residual superAg-specific cells. However, responses to superAgs could represent a special case, and for this reason we undertook to test the effects of CsA on T cell responses to other stimuli, both *in vivo* and *in vitro*. We also compared the effects of CsA to those of two other potent immunosuppressive agents, rapamycin (RAPA) and FK520 (henceforth referred to as FK). The latter drug is a close analogue of FK506 that binds the same immunophilins and has similar immunosuppressive properties (2).

CsA markedly enhanced the depletion of $CD4^+$ T cells, in mice treated with anti-TcRαß MoAbs. Moreover, in CsA-treated mice, almost all residual T cells showed severe and prolonged TcR downmodulation. In contrast, while CsA also promoted the depletion of superAg reactive T cells (primarily of the $CD4^+$ phenotype), this was not accompanied by any apparent TcR downmodulation. Unlike CsA, RAPA and FK had no effects on the degree of peripheral T cell deletion. Similarly, of the three drugs tested, only CsA inhibited superAg-induced peripheral T cell anergy.

In vitro, CsA enhanced apoptosis of anti-TcR-stimulated T cells. T cell elimination was most marked in APC-depleted populations, excluding phagocytosis as a mechanism, and occurred in the absence of proliferation.

While the mechanisms of action of CsA on deletion and anergy have not been elucidated, our experiments exclude some possibilities. Since FK has no effects on peripheral deletion, it appears that the calcium-dependent calcineurin pathway (3-7) is not involved. CsA inhibits CD3 MoAb-mediated apoptosis in the thymus (8), possibly by blocking a calcium-dependent signal. Therefore, our results support the view that peripheral T cell and thymocytic apoptosis occur by different mechanisms. However, there is also a CsA-resistant pathway in the thymus, since CsA delays but does not prevent clonal deletion (9-11). It is unclear if CsA-induced alterations in peripheral anergy and deletion are linked. Our results suggest that the effects of CsA on peripheral T cells may be relevant to the development of tolerance in some models.

3. Materials and Methods

Mice. BALB/c mice (H-2^d, Mls-1^b), DBA/2 mice (H-2^d, Mls-1^a), C57BL/10 (B10) mice (H-2^b, Mls-1^b) and Wistar Furth (WF) rats were purchased from Harlan Sprague Dawley (Indianapolis). Adult B10 mice (4-6 wk old) were thymectomized in our facility as described (12) and rested for at least 7 d before the onset of treatments.

MoAbs and Reagents. PE-conjugated anti-CD4, and PE-conjugated anti-CD8 were purchased from Cedarlane Laboratories (Hornby, Canada). The following MoAbs were produced as culture supernatants and purified by protein G (Pharmacia, Montreal, Quebec, Canada) affinity chromatography: J11D (ATCC, Rockville, Maryland), anti-Vß6 (44-22-1 hybridoma; 13), anti-Vß8.1-8.2 (KJ16 hybridoma; 14), anti-Vß14 (14.2 hybridoma; 15), anti-TcRαß (H57-579 hybridoma; 16), Thy 1.2 (ATCC) and anti-mouse-CD3 (hybridoma 145-2C-11; 17). For flow cytometry analysis, the purified MoAbs were directly conjugated to FITC. CsA was a gift of Sandoz (Dorval, Canada). RAPA was a gift of Wyeth-Ayerst Research, Saint-Laurent, Quebec, Canada. FK520 (L-683,590) was a gift of Merck Research Laboratories, Rahway, N.J. SEB, Phorbol myristate acetate (PMA) and ionomycin were purchased from Sigma Chemical Co. (St. Louis, MO).

Immunizations, MoAb and drug Treatments. SuperAg immunizations were performed as described (1 [chapter III]), by tail vein injections of either 10^7 Mls-1^{a+} (MMTV-7^+) splenocytes (RBC depleted), or 50 μg of SEB. MoAb treatments were performed by injecting 50-100 μg purified MoAb *i.v.*. Cells, SEB or MoAb were injected in a volume of 0.25 ml of PBS. The day of superAg injection or MoAb treatment was considered day 0 of our experiments. CsA was suspended in olive oil (OO)(20 mg/ml) and given intraperitoneally at a dose of 50 mg/kd/d, unless otherwise indicated. RAPA and FK520 were dissolved in ethanol (20%) and cremophor (80%) and injected at doses of 0.2-2.0 mg/kg/d and 0.1 - 10 mg/kg/d, respectively, using the same protocol as for CsA administration. Drugs were administered daily starting 1 d before immunization or MoAb treatment (day -1) and continued until the day before the mice were killed, unless otherwise indicated. Control groups included untreated mice, drug treatment only, immunized or MoAb-treated mice (no drug), and diluent-treated mice. In control mice, drug and/or diluent were administered in the same dose and/or volume as in the experimental groups.

Xenogeneic cell immunizations. These experiments were performed as described above for Mls-1^a immunization, except that instead of mouse cells recipients were treated with 10^7- 10^8 WF rat splenocytes (injected *i.v.*).

Flow Cytometry Analysis. For two-color flow cytometry analysis, LN cells (pooled cervical, axillary, para-aortic, and mesenteric LN) were stained with either PE-anti-CD4 or PE-anti-CD8, and with one of the FITC labelled-anti-Vß or other MoAbs mentioned above. One-color flow cytometry analysis was performed with FITC-labelled MoAbs only. Briefly, 5 x 10^5 LN cells were incubated with PE-conjugated MoAb for 15 min at 4°C, then washed three times with PBS containing 1% FCS (Gibco, Burlington, Canada) and then incubated in the same conditions with the second FITC-labelled-anti-Vß MoAb. Propidium iodide (Sigma Chemical Co.) was added after the last wash. Dead cells were excluded based on propidium iodide staining and forward scatter. In each sample, 10^4 cells were analyzed on a FACScan^R (Becton Dickinson & Co., Mississauga, Canada). Statistical analyses were performed with the Student's t test.

In vitro Proliferative Assays. LN cells were cultured in 96-well flat-bottomed plates (Gibco Laboratories, Grand Island, NY) in quadruplicates at 5 x 10^5 cells/well with either: (a) for SEB stimulation, with 10^6 syngeneic irradiated (2,000 rad) splenocytes of untreated BALB/c mice and 10 μg/ml of SEB, or 1 μg/ml of SEA as a control; or (b) for Mls-1^a experiments, with 10^6 irradiated (1,000 rad) DBA/2 splenocytes or irradiated (2,000 rad) allogeneic C3H/HeJ (H-2^k, Mls-1^b) splenocytes. Prior to *in vitro* stimulation with MoAb, tissue culture wells were coated with MoAb. Where indicated, PMA was added to cultures at

a concentration of 10 ng/ml, ionomycin was added at a concentration of 0.4 μg/ml, and Con A was added at a concentration of 5μg/ml. Cells were cultured in RPMI 1640 (Gibco Laboratories) containing 10% FCS, L-glutamine, 5 x 10^{-5} M 2-ME, and antibiotics. Cells were pulsed with [^{3}H]thymidine (ICN, Montreal, Canada) at day 3 of culture and harvested on day 4, unless otherwise indicated. Days in culture refer to the day that the cells were harvested. In all cases, the cells were harvested after an 18-h incubation with [^{3}H]thymidine. For stimulation with SEB, anti-TcRαß MoAb, PMA + ionomycin, and Con A, Δcpm = cpm experimental (with stimulus) - cpm control (no stimulus). In the case of Mls-1^a or MLC stimulation, Δcpm = cpm experimental - cpm with syngeneic irradiated stimulator cells.

Flow cytometry analysis of cultured cells. Cultures were performed in 24-well plates (Gibco) and consisted of 4 x 10^6 purified T cells, with or without 1.2 x 10^6 T cell-depleted irradiated (2000 rads) APCs, and with or without various stimulating agents (as described above). Cells were recovered after 48 hours in culture, and transferred to non-antibody-coated wells overnight. The next day, dead cells were removed on a lymphocyte-M gradient (Cedarlane, Toronto, Canada), and cells were stained and analyzed as described above.

Cell death and apoptosis assays. Cell death in culture was counted by trypan

blue dye staining. Apoptosis was detected as described by Perandones et al (18). Briefly, cells were harvested, centrifuged and resuspended in 400 μl hypotonic lysis buffer (0.2% Triton X-100, 10 mM Tris, 1mM EDTA, pH = 8) and centrifuged 15 min at 13,800 g. The supernatant (SN), was separated immediately; half was used for agarose gel electrophoresis, and the other half, as well as the pellet containing large fragments of DNA, was used in the diphenylamine (DPA) assay. For the latter assay, Perchloric acid (0.5M) was added to the pellets containing large MW DNA, and to the SN containing DNA fragments, followed by 2 volumes of a solution containing 0.088 M DPA, 98% v/v glacial acetic acid, 1.5% v/v sulfuric acid and 0.5% v/v of 1.6% acetaldehyde solution. The samples were stored at 4°C for 48 h. The colorimetric reaction was quantitated on an ELISA reader at OD 575 nm, and the percentage of DNA fragmentation was expressed as follows:

$$\% \text{ DNA fragmentation} = \frac{(\text{OD SN}) \times 100}{(\text{OD SN}) + (\text{OD pellet})}$$

Cell depletion. In some experiments, LN B cells were depleted by panning on anti-Ig-coated plates as described (19). In some experiments, macrophage and B-cell depletion was accomplished by removal of adherent cells by incubation at 37°C on petri dishes (2 passages), followed by 2 passages on Cellect mouse T cell immunocolumns (Biotex Laboratories Inc., Edmonton, Alberta, Canada). The

latter method resulted in cell populations that were 98-99% T cells, as determined by flow cytometry, and that failed to proliferate *in vitro* in response to SEB. Purified APCs were prepared from spleen cells by depleting T cells by panning, with 2 passages on CD3 MoAb-coated petri dishes, followed by irradiation of cells.

4. Results

CsA enhances CD4$^+$ T cell deletion in mice treated with anti-TcR αβ MoAb.

Treatment of BALB/c mice with anti-TcR αß MoAb (H57-579) caused a decline of LN TcRαß$^+$ cells from a mean of 80.7% to 47.4% (Table 1; Fig. 1A, 1B). This resulted primarily from a loss of CD4$^+$ T cells, from 60.0% to 25.6%, with only a small decrease in the percent CD8$^+$ T cells. Administration of CsA for 7 days (day -1 to 6) in combination with anti-TcR MoAb (day 0) resulted in markedly increased CD4$^+$ T cell deletion (Table 1, Fig. 1B), such that a mean of only 10.1% of LN T cells expressed that phenotype ($p < 0.005$ vs diluent-treated mice), but with no increase in the deletion of CD8$^+$ cells. The decline in T cell numbers was accompanied by a corresponding decline of Thy-1$^+$ cells, and a relative increase in J11D$^+$ cells (B cells), confirming that the loss was due to T cell deletion rather than CD4 downmodulation. The administration of CsA alone for 7 to 21 days did not cause a significant decline in T cell numbers. Interestingly, TcRαß expression was severely depressed in anti-TcRαß MoAb and CsA-treated mice, as detected by flow cytometry with both a CD3 MoAb (Fig. 1, B and D), and anti-TcRαß MoAb (not shown). In mice that received CsA from day -1 to day 6, CD3-TcRαß expression was not restored 3 days after withdrawal of CsA (not shown), but was restored 15 days post withdrawal (Table 1). In the latter case, there was a residual effect of CsA, but CD4$^+$ T cell numbers were

TABLE 1. Increased T cell deletion and TcR downmodulation with anti-TcRαß MoAb and CsA treatment

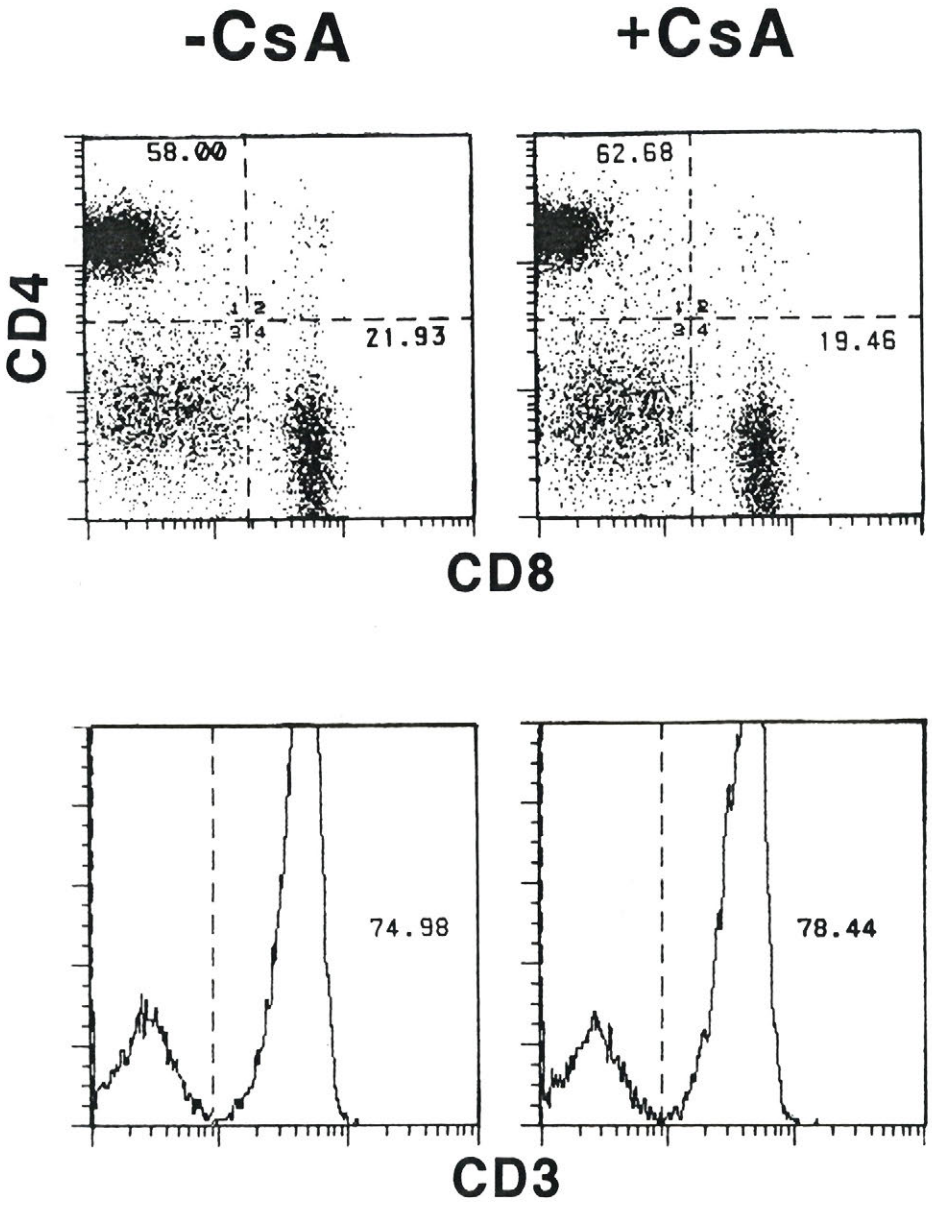
TcRαß MoAb	CsA (days)	Day of Analysis	CD4$^+$	CD8$^+$	J11D$^+$	CD3$^+$	TcRαß	Thy1$^+$
-[a]	-		60.0±3.1[b]	19.7±1.8	19.1±3.7	79.5±2.8	80.7±4.4	77.0±5.6
+[c]	-	7	25.6±4.9	17.8±1.9	39.2±1.4	44.3±3.6	47.4±3.6	45.1±3.0
+	-1 to 6[d]	7	10.1±2.1	27.8±2.1	52.6±2.4	1.8±0.2	0.3±0.1	33.5±3.7
+	-1 to 6	21	23.2±5.2	21.3±2.9	43.9±3.9	43.1±3.9	41.1±3.4	ND
+	-	20	35.6±3.8	12.6±1.2	36.4±4.3	53.3±4.1	47.9±1.4	48.3±3.8
+	-1 to 19	20	10.0±4.2	21.5±3.6	55.2±2.7	28.5±14.8	31.0±15.5	30.1±2.8
+(x2)[e]	-	20	31.7±3.5	13.0±2.0	44.0±2.0	42.3±8.0	43.3±1.5	43.5±1.7
+(x2)	-1 to 19	20	7.7±0.6	14.1±2.6	66.3±2.9	1.4±0.5	0.9±0.5	22.0±6.5

a. -, not administered.
b. Results represent the percent positive LN cells (±1 S.D.).
c. +, 50 μg anti-TcRαß MoAb administered *i.v.* on day 0.
d. 50 mg/kg/d of CsA administered *i.p.*, over indicated time period. The administration of CsA for 7 to 21 days without anti-TcRαß MoAb treatment did not cause a decline in T cell numbers (data not shown)
e. + (x 2), 50 μg anti-TcRαß MoAb administered *i.v.* on days 0 and 14.

Figure 1. Enhanced $CD4^+$ T cell deletion and TcR downmodulation in CsA/anti-TcR MoAb treated BALB/c mice. (A) Two-color CD4/CD8 staining, and one-color CD3 staining, in untreated mice (left upper and lower histograms), or CsA treated mice (7 day treatment with 50 mg/kg/d, right upper and lower histograms). (B) Cells were analyzed on day 7 in mice treated with 50 μg anti-TcRαß MoAb (day 0) without CsA (left upper and lower histograms), or with CsA from day -1 to day 6 (right upper and lower histograms). Upper histograms represent CD4/CD8 two-color staining, while lower histograms represent J11D/CD3 two-color staining. (C) Same experimental conditions as in Fig. 1B, but mice were treated with CsA from day -1 to 19, and cells were analyzed on day 20. (D) Cells were analyzed on day 20 in mice that received 50 μg anti-TcRαß MoAb on days 0 and 14, without CsA (left upper and lower histograms), or with CsA from day -1 to day to 19 (right upper and lower histograms). Staining was performed as in Fig. 1A. In all histograms, the numbers listed represent the percentage of positive cells.

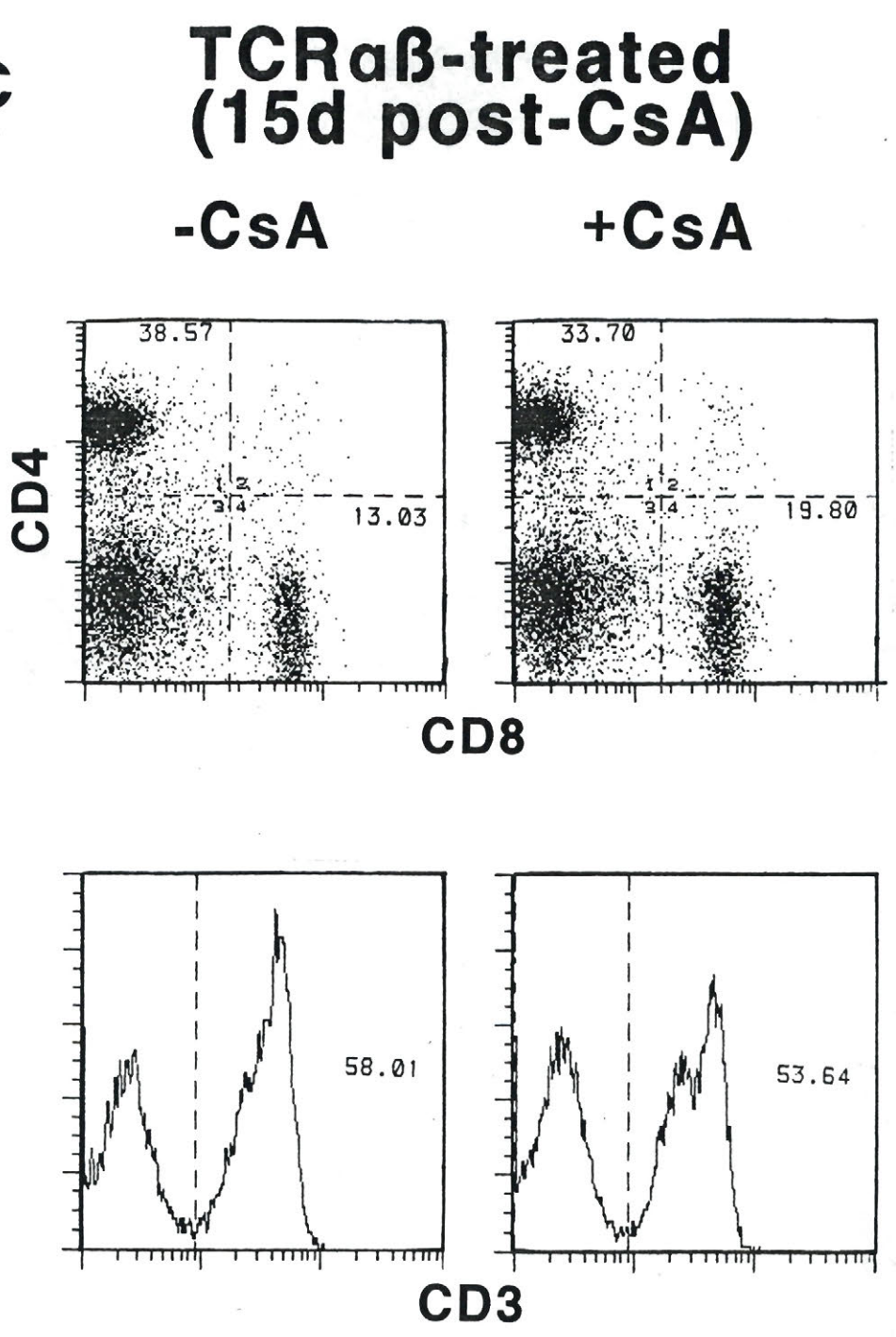
A

Controls



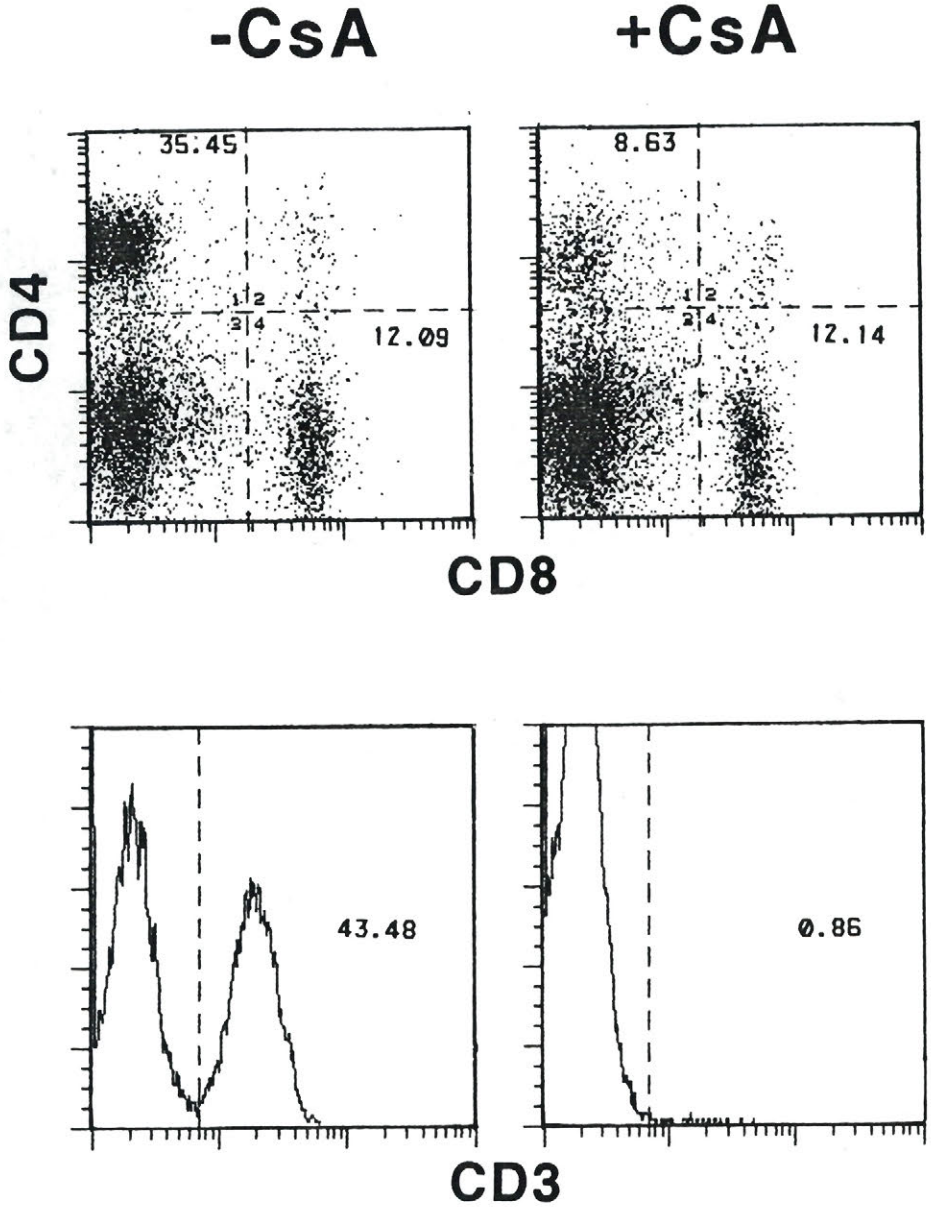
C

TCRαß-treated (15d post-CsA)



D

TCRαß-treated (d20 CsA)



much increased. In mice that received only one antibody injection (day 0), and continuous CsA therapy, there was gradual re-expression of the TcR, such that by day 20 the density of expression was approximately 70% as high as in non-CsA-treated mice (Fig. 1C), although $CD4^+$ T cell numbers remained severely depressed (mean of 10.0%). However, when mice were treated continuously with CsA from day -1 to day 19, and received injections of anti-TcRαß MoAb on days 0 and 14, there was persistent downmodulation of the TcR at day 20 (Fig. 1D), and a further deletion of $CD4^+$ cells (mean of 7.7%). These results suggest that the maintenance of maximal T cell depletion requires continuous CsA treatment, and that CsA-induced TcR downmodulation is probably dependent on the persistence of circulating anti-TcR MoAb. In fact, TcR expression was restored within 16hrs in culture (data not shown).

Functional activity

LN cells were depleted of B cells by panning and tested for response to Con A, matrix-bound anti-TcRαß MoAbs, and PMA + ionomycin. Anti-TcRαß MoAb treatment did not induce a state of anergy, and responses were only slightly reduced by the administration of CsA (Table 2), presumably due to decreased $CD4^+$ T cell numbers. The response to anti-TcRαß MoAb stimulation is consistent with rapid re-expression of the TcR *in vitro*, as mentioned above.

TABLE 2. B Cell-Depleted LN-Lymphocyte Responses

In Vivo Treatment		Proliferative Response (cpm x10^{-3})			
anti-TcR$\alpha\beta$ MoAb[a]	CsA[b]	BKG[c]	anti-TcR$\alpha\beta$ MoAb	PMA + Ionomycin	Con A
-[d]	-	0.25±0.09[e]	89±12	199±41	73±8
+	-	0.44±0.15	100±17	158±17	40±12
+	+	0.22±0.07	47±20	144±45	31±14

a. Mice were injected (*i.v.*) with 50 μg of anti-TcR$\alpha\beta$ MoAb on days 0 and 14.
b. Mice were treated with 50 mg/kg/d of CsA (*i.p.*) from day -1 to day 19, and LNs were recovered on day 20. B cells were depleted by panning. Similar results were obtained when LNs were recovered on day 7 (after only one MoAb injection) (data not shown).
c. BKG, background proliferation.
d. -, untreated; +, treated.

Effects of CsA, RAPA and FK on T cell deletion in superantigen-immunized mice.

We previously reported that CsA enhances the partial deletion of superantigen-reactive T cells in mice immunized with SEB or Mls-1^a (1 [chapter III]). Unlike deletion with anti-TcR MoAb, this CsA/superAg-induced deletion was not accompanied with TcR downmodulation, since there was no discrepancy between the numbers of T cells expressing either TcR$\alpha\beta$, Thy-1, or the sum of CD4$^+$ and CD8$^+$ cells (1 [chapter III]). Moreover, there was no change in TcR-Vβ frequencies after *in vitro* culture (our unpublished observations). In this study, we compared the effects of CsA, RAPA, and FK on this deletion process. Mls-1^a induces deletion of Vβ6$^+$ T cells (1 [chapter III],20), while SEB induces deletion of Vβ8$^+$ T cells (1 [chapter III],24). Unlike CsA, RAPA and FK had no effects on T cell deletion in mice treated with either Mls-1^a (Fig. 2,3) or SEB (Fig. 4). This held true even when RAPA and FK were administered at the very high immunosuppressive doses of 2 mg/kg/d and 10 mg/kg/d, respectively.

Effects of CsA and RAPA on anergy

CsA, as we previously reported (1 [chapter III]), inhibits superAg-induced T cell anergy. This type of anergy is observed in T cells that resist deletion (1 [chapter III], 22-24). The effects of CsA on anergy is most apparent at moderate doses (12.5 mg/kg/d), since at high doses (50 mg/kg/d) this drug stimulates

Figure 2. TcR-Vß6^{+} T cell deletion in Mls-1^{a} (MMTV-7) immunized mice. Representative CD4 vs Vß6 two-color histograms of BALB/c mice treated with: (A) Mls-1^{a} immunization (DBA/2 donor splenocytes); (B) Mls-1^{a} immunization and RAPA treatment of 2 mg/kg/d (similar results were obtained at RAPA doses ranging from 0.2 to 2.0 mg/kg/d); (C) Mls-1^{a} immunization and FK treatment at 10 mg/kg/d (similar results were obtained at FK doses of 0.1 to 10 mg/kg/d, while higher doses were toxic); (D) Mls-1^{a} immunization and CsA treatment (50 mg/kg/d). In all cases, the mice were immunized with Mls-1^{a} cells on day 0, treated with drugs i.p. from day -1 to day 13, and LN were recovered and analyzed on day 14.

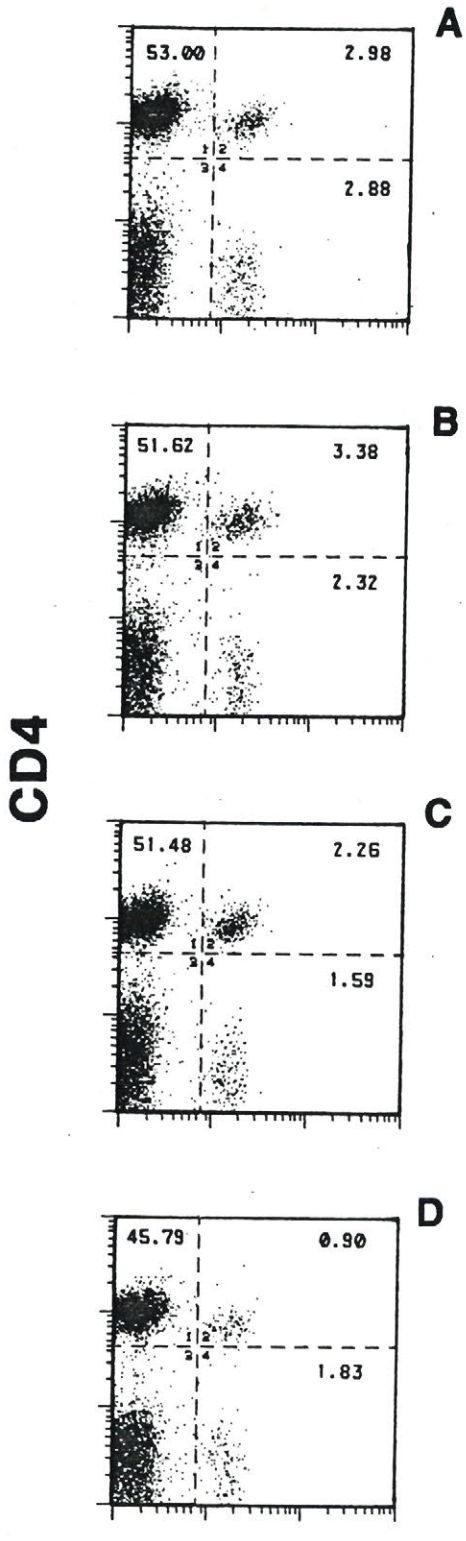

A
53.00
2.98
2.88
B
51.62
3.38
2.32
C
51.48
2.26
1.59
D
45.79
0.90
1.83
CD4
Vß6

Figure 3. CsA, but not RAPA and FK, enhances deletion of Vß6$^+$ T cells in Mls-1^a immunized mice. BALB/c mice were immunized with Mls-1^a cells and treated with immunosuppressive drugs as described in Fig. 2. The results represent the mean percent of (± 1SD) of TcR-Vß6$^+$ T cells among CD4$^+$ T cells (hatched bars) or TcR-Vß14$^+$ T cells among CD4$^+$ T cells (dotted bars), in groups of 3 to 5 mice treated with olive oil (OO), CsA, RAPA and FK as indicated. LN cells were recovered at day 14 and analyzed by two-color flow cytometry. In untreated BALB/c mice, there were 10.0 ± 0.2% Vß6$^+$ cells and 10.2 ± 0.1% Vß14$^+$ T cells among CD4$^+$ T cells (not shown). Only CsA enhanced deletion ($p < 0.0001$ vs diluent-treated mice) in Mls-1^a-immunized mice.

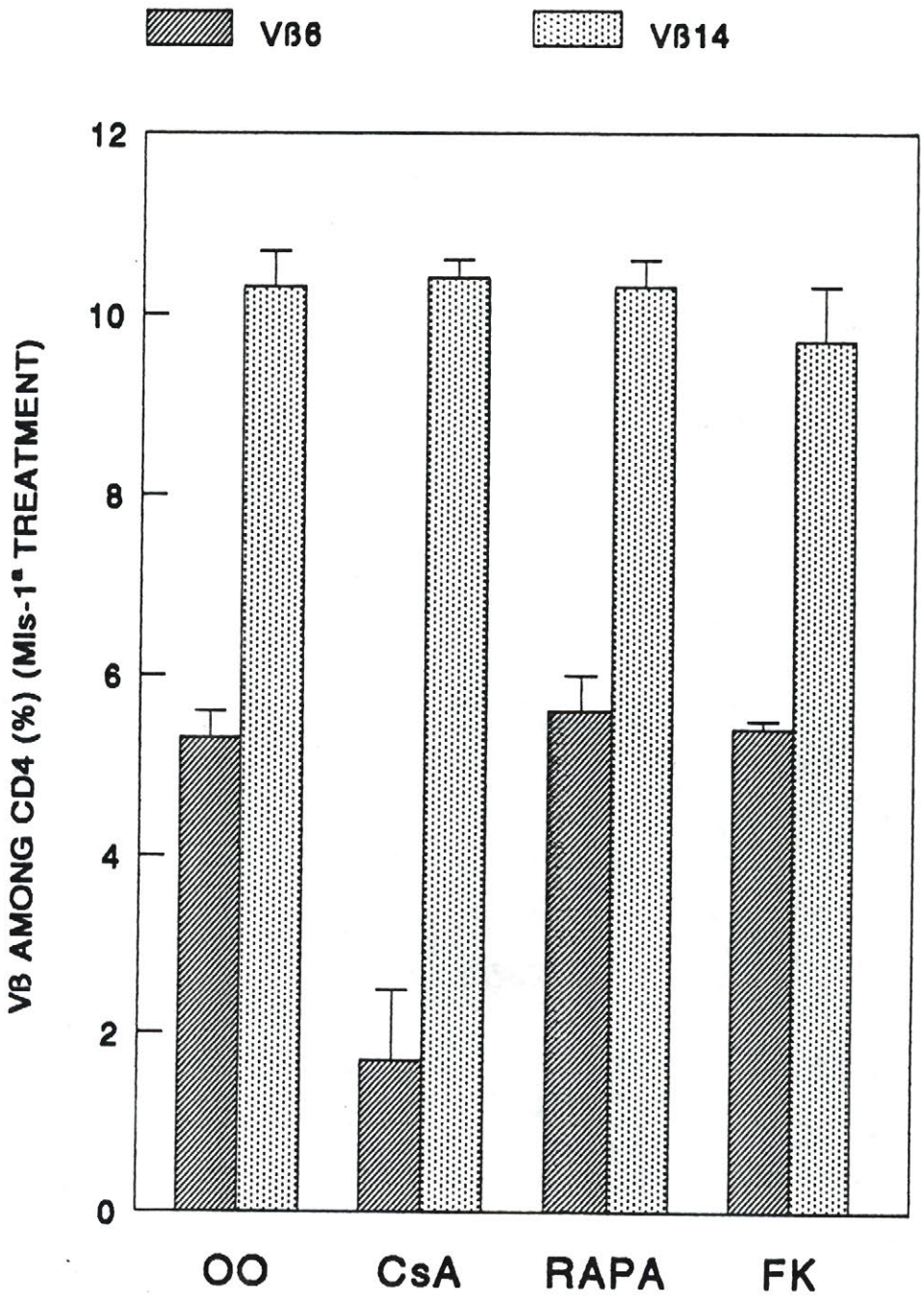
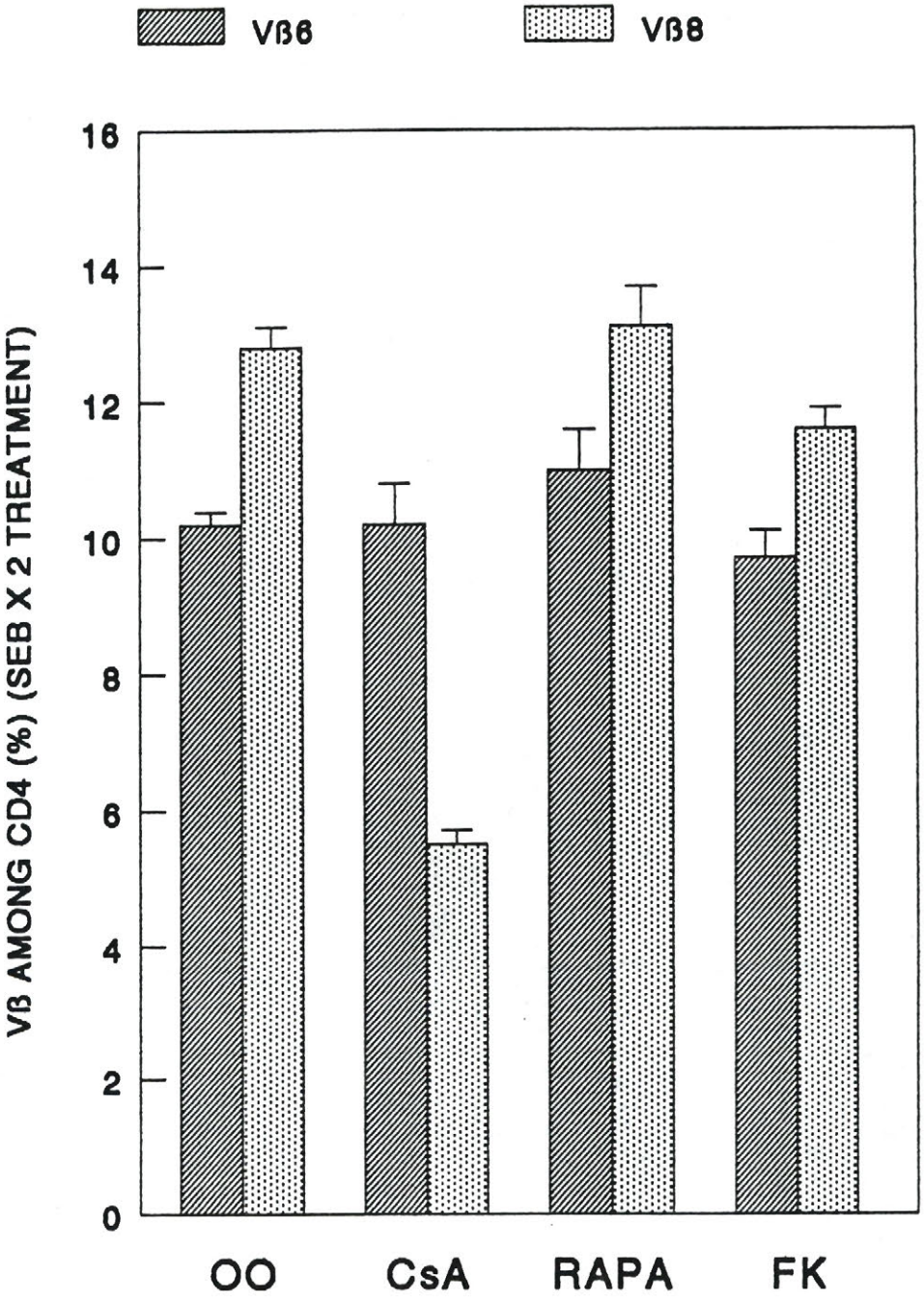

Vß6
Vß14
12
10
8
6
4
2
0
Vß AMONG CD4 (%) (Mls-1ª TREATMENT)
OO
CsA
RAPA
FK

Figure 4. CsA, unlike RAPA and FK, enhances the deletion of SEB-reactive Vß8$^+$ T cells. BALB/c mice received injections of SEB (50 μg, injected *i.v.*) on days 0 and 4, and were treated with vehicle (OO), CsA, RAPA, or FK from day -1 to 7 (same doses as in Fig. 2). LN cells were recovered and analyzed by two-color flow cytometry on day 8. The results represent the mean percent ($\pm$ 1SD) of TcR-Vß6$^+$ T cells among CD4$^+$ T cells (hatched bars), or TcR-Vß8$^+$ T cells among CD4+ T cells (dotted bars). 3-5 mice were analyzed in each group. In untreated BALB/c mice, there were 19.3 $\pm$ 0.2% Vß8$^+$ cells among CD4$^+$ cells (not shown). Only CsA enhanced deletion ($p < 0.0001$ vs diluent-treated mice).


Vß6
Vß8
16
14
12
10
8
6
4
2
0
Vß AMONG CD4 (%) (SEB X 2 TREATMENT)
OO
CsA
RAPA
FK

extensive clonal deletion, as mentioned above. In similar experiments, we now show that FK and RAPAadministered at high immunosuppressive doses have no effect on the induction of anergy (Fig. 5, A and B). The administration of these drugs at low or moderate doses also failed to alter the induction of anergy (data not shown).

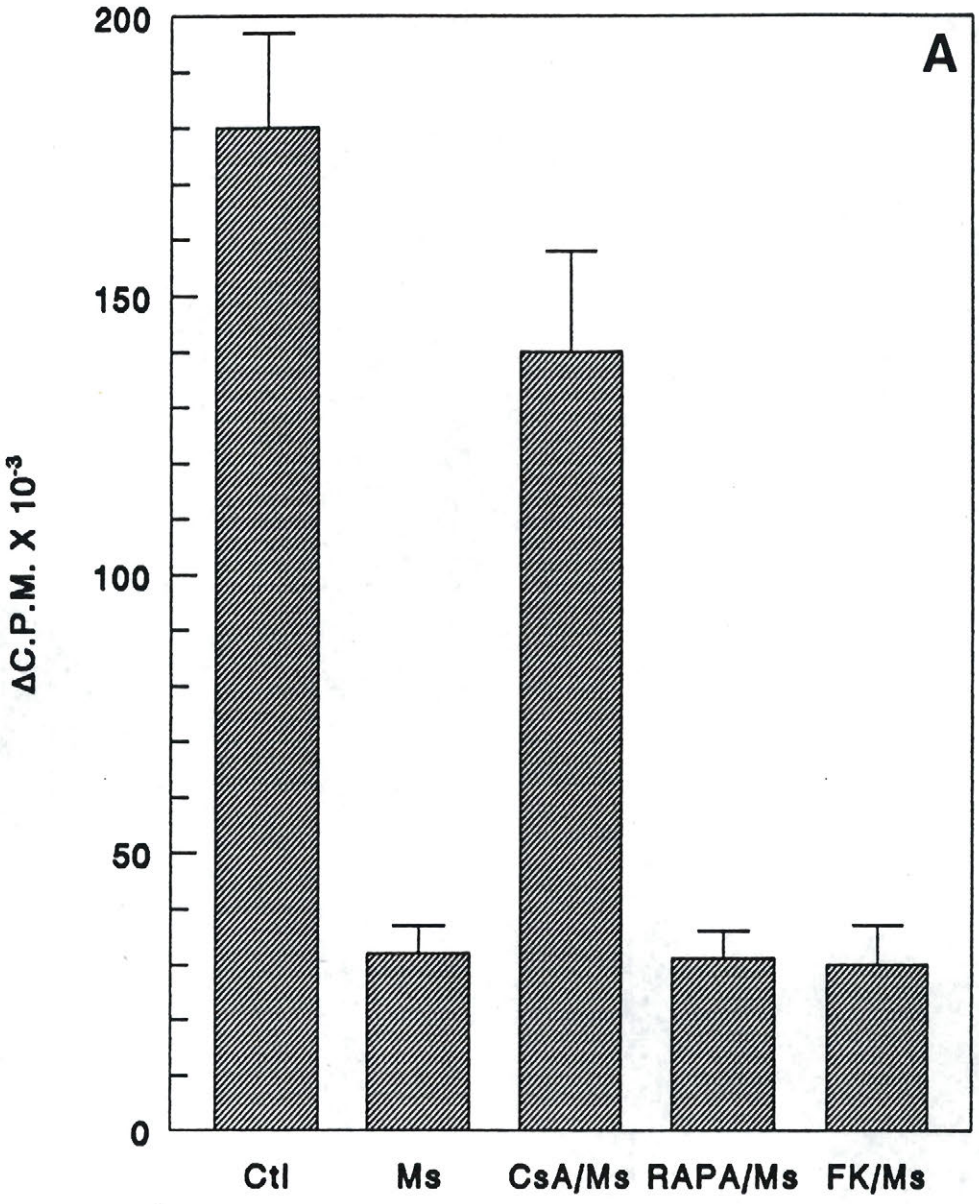
Clonal deletion in response to xenogeneic cells.

The work of Ildstad et al. (25) demonstrates that T cells bearing some Vß elements are deleted in mixed bone marrow chimeras (mouse + rat into mouse). This type of deletion probably occurs in the thymus. To develop a transplantation model of peripheral T cell deletion, we tested the effects of injecting WF rat spleen cells into thymectomized B10 mice. Our results (Fig. 6) reveal that the rat cells induce a moderate deletion of Vß11$^+$ T cells. The nature of the antigen(s) mediating this effect is unknown. In this transplantation model, CsA did not increase peripheral T cell deletion (Fig. 6).

In vitro apoptosis.

Cell death was analyzed in culture over periods of 24 to 72 h. To quantitate the percent of apoptotic cells we used a colorimetric method as described by Perandones et al (18). Purified T cells, or unseparated LN cells, were stimulated with either anti-TcRαß MoAb, anti-Vß8.1, 8.2 MoAb (KJ16), or SEB. At 24hrs

Figure 5. CsA, unlike RAPA and FK, inhibits anergy induction in superAg-immunized mice. (A) BALB/c mice were either untreated controls (Ctl), or were immunized with Mls-1^a cells (Ms) with or without drug treatment (CsA, RAPA, or FK), as explained in the legend to Fig. 2 with the exception that CsA was given at a dose of 12.5 mg/kg/d (a dose that does not augment TcR-Vß6 deletion (1)). LN cells were recovered at day 14 and stimulated in MLC with DBA/2 irradiated spleen cells (Mls-1^a stimulator cells). The results represent the mean Δcpm ($\pm$ 1SD) in groups of 3-5 mice. (B) BALB/c mice were either untreated (Ctl) or immunized with SEB (x2) with or without either CsA or FK treatment (as described in the legend to Fig. 4) from day -1 to 11. LN cells were recovered at day 12 and stimulated in vitro with SEB. The results represent the mean Δcpm ($\pm$ 1SD) in groups of 5 mice.


A
200
150
100
50
0
ΔC.P.M. X 10-3
Ctl
Ms
CsA/Ms
RAPA/Ms
FK/Ms

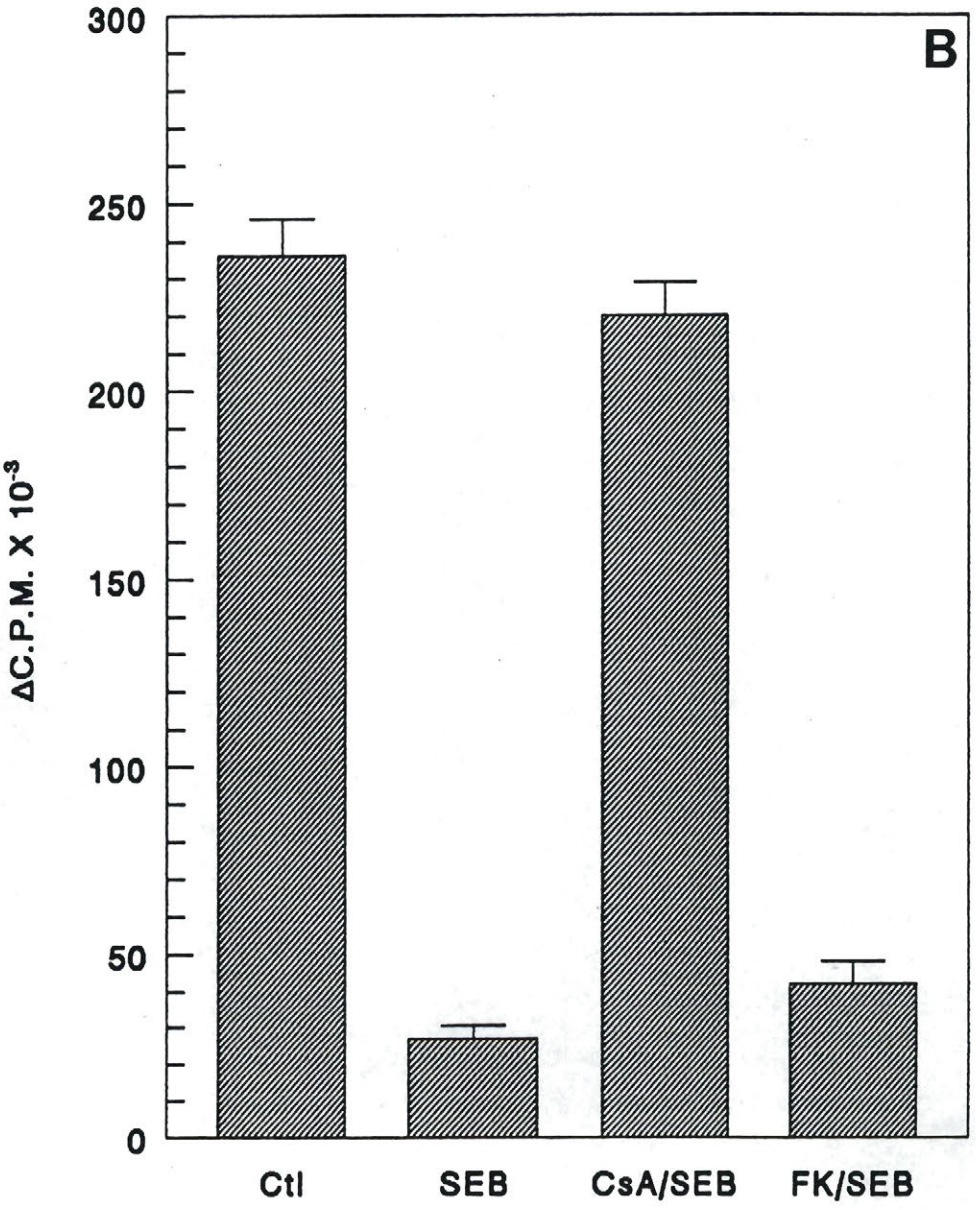
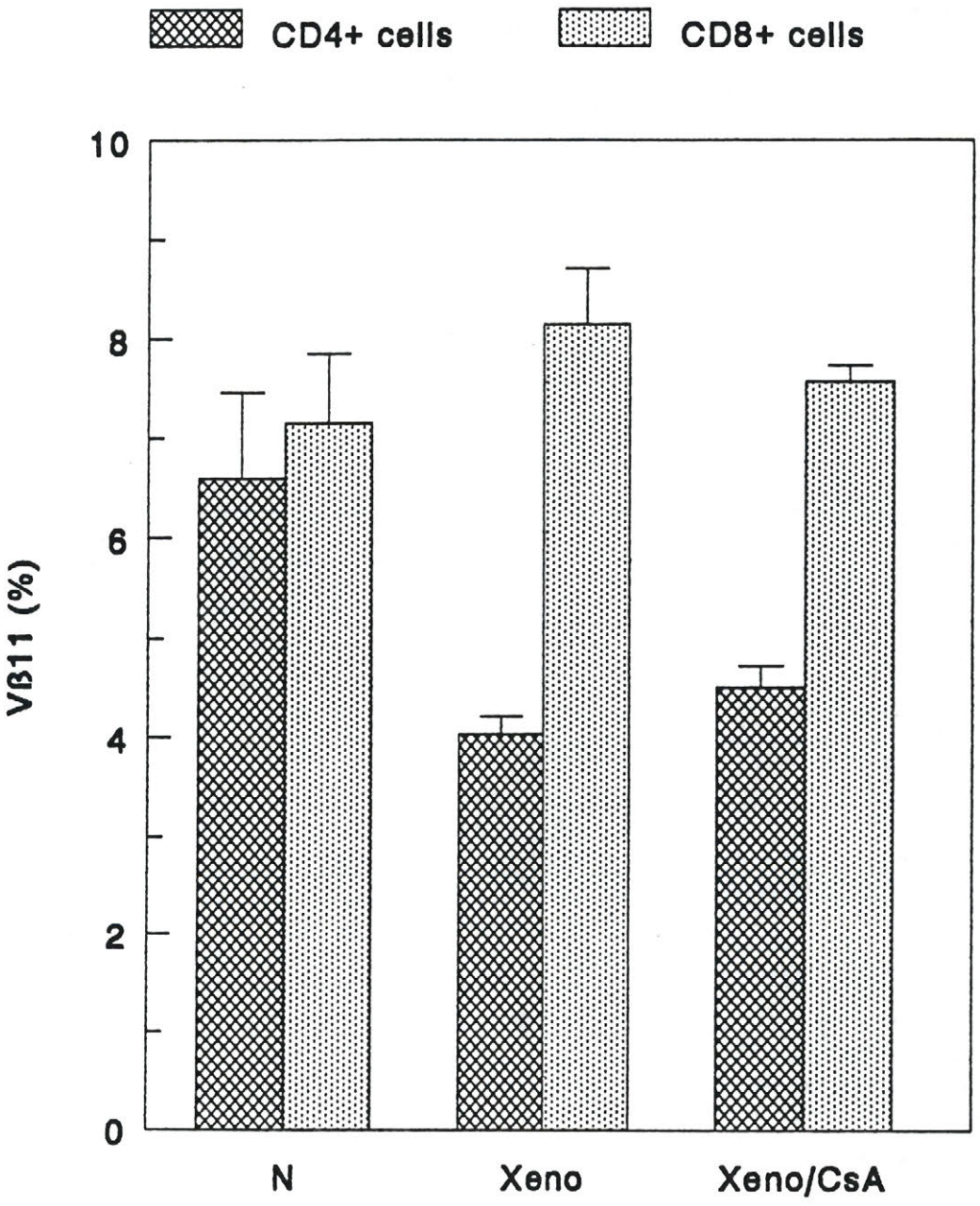

B
300
250
200
150
100
50
0
ΔC.P.M. X 10-3
Ctl
SEB
CsA/SEB
FK/SEB

Figure 6. CsA does not enhance Vß11$^+$ T cell deletion in B10 mice injected with WF rat spleen cells. Results represent the mean percent Vß11$^+$ LN T cells (± 1SD) in groups of 5-6 mice treated as follows: N (normal B10 mice); Xeno, B10 mice injected *i.v.* with rat spleen cells (day 0); Xeno/CsA, B10 mice injected *i.v.* with rat spleen cells (day 0), and treated with CsA (50 mg/kg/d) from day -1 to day 9. LN cells were recovered on day 10 and analyzed by two-color flow cytometry. The *i.v.* injection of WF spleen cells in B10 mice provoked a partial deletion of CD4$^+$/Vß11$^+$ T cells (cross-hatched bars), but not CD8$^+$/Vß11$^+$ T cells (dotted bars). Deletion was not enhanced by CsA.


CD4+ cells
CD8+ cells
10
8
6
4
2
0
Vβ11 (%)
N
Xeno
Xeno/CsA

in culture, CsA had no effects on cell death (not shown). At latter time points, spontaneous cell death in CsA-free cultures ranged from 15% to 30% (data not shown), as previously reported (18). In non-stimulated populations, cell death was not increased by the addition of CsA (Fig. 7). On the other hand, the addition of CsA to anti-TcRαß-stimulated T cells resulted in a marked increase in apoptotic cell death, which was up to 35% greater than control cultures (Fig. 7). Gel electrophoresis of DNA revealed a ladder pattern typical of apoptosis, but this was also present in CsA-free cultures, reflecting the high rate of spontaneous apoptosis (data not shown). When CsA was added to cultures stimulated *in vitro* with KJ16, there was a partial deletion of $CD4^+$ TcR-$V\beta8^+$ T cells, that was most marked in the absence of APCs (Fig. 8). RAPA did not provoke this type of deletion. The disappearance of $V\beta8^+$ T cells with KJ16 was not due to adherence of cells to culture wells, or to TcR downmodulation. Cells were removed from culture, washed and reincubated in non-antibody-coated dishes overnight prior to staining, and we did not detect $CD4^+$ $TcR\alpha\beta^-$ or $CD4^+$ $CD3^-$ T cells by two-color flow cytometry (data not shown). Moreover, no deletion was observed in cells stimulated with KJ16 in the absence of CsA (Fig. 8). RAPA and CsA prevented proliferation of stimulated $V\beta8^+$ T cells equally. Therefore, lack of proliferation does not explain the low numbers of $V\beta8^+$ T cells in CsA-supplemented cultures. Moreover, loss of $V\beta8^+$ T cells occurred in highly purified T cell populations that did not proliferate in response to KJ16 MoAb, even without CsA. Therefore,

Figure 7. CsA enhances apoptosis in T cells stimulated with anti-TcRαß MoAb *in vitro*. CsA (1000 ng/ml) was added to cultures of unstimulated (control), or matrix-bound anti-TcRαß-stimulated (anti-TcR) BALB/c splenic T cells. The results represent the increase in the percent (± 1SD) of apoptic cells (above spontaneous apoptosis, see text), as determined with the DPA assay, when CsA was added to cultures that were incubated for 48 h (hatched bars), or 72 h (dotted bars). The results of a representative experiment are shown, and a total of 3 experiments yielded similar results.

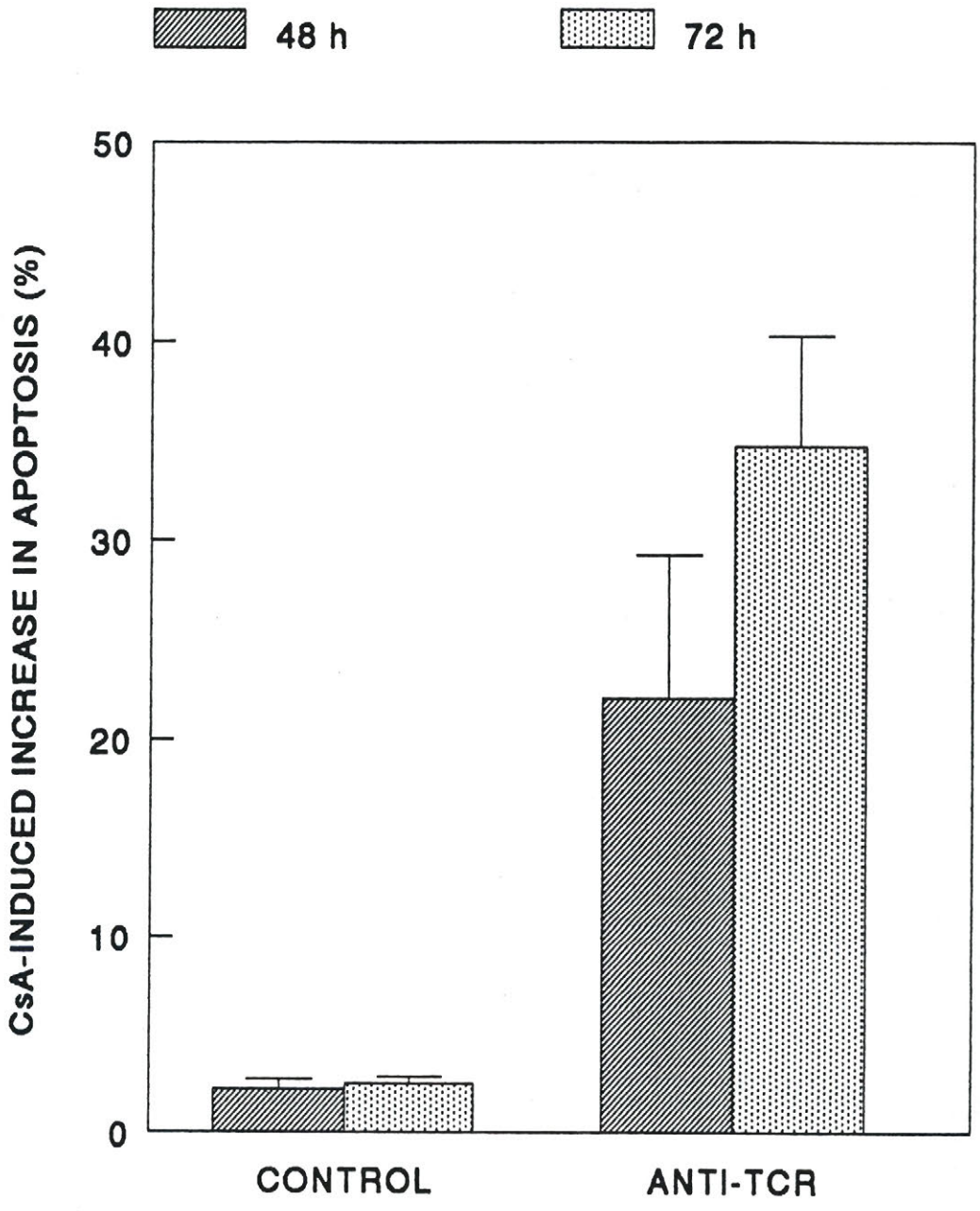
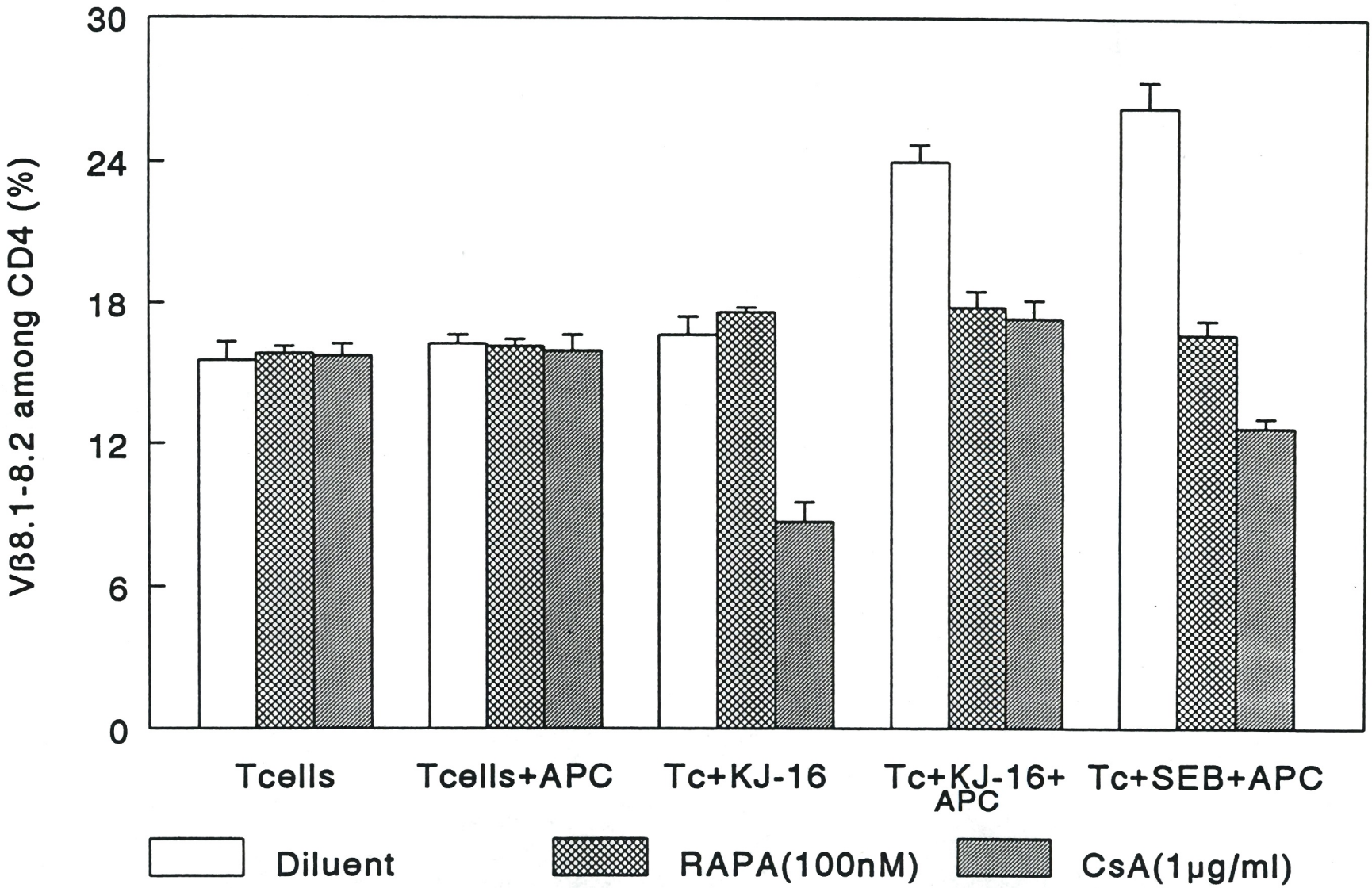

48 h
72 h
CsA-INDUCED INCREASE IN APOPTOSIS (%)
50
40
30
20
10
0
CONTROL
ANTI-TCR

Figure 8. Deletion of KJ16-stimulated LN cells *in vitro*. The LN cells of normal BALB/c mice were highly enriched for T cells by depleting macrophages and B cells. The T cells were cultured in medium containing diluent (open bars), CsA (1000 ng/ml, hatched bars), or RAPA (100 nM, cross-hatched bars). The following cultures were analyzed: T cells (Tc); Tc + APC (irradiated T cell-depleted syngeneic spleen cells); Tc + KJ16, Tc stimulated with the KJ16 MoAb (anti-TcR-Vß8.1-8.2); Tc + KJ16 + APC; Tc + SEB + APC. The cells were recovered after 48 h in culture, transferred into non-antibody-coated wells overnight, and live cells were analyzed for CD4 and Vß8.1-8.2 expression by two-color flow cytometry. The results of a representative experiment are shown. A total of 3 experiments yielded similar results. SEB + Tc (without APC) did not result in $KJ16^+$ T cell deletion (not shown).


30
24
18
12
6
0
Vβ8.1-8.2 among CD4 (%)
Tcells
Tcells+APC
Tc+KJ-16
Tc+KJ-16+ APC
Tc+SEB+APC
Diluent
RAPA(100nM)
CsA(1μg/ml)

proliferation need not precede apoptosis in this model. The results suggest that deletion is not mediated by phagocytosis of antibody-coated T cells, since it occurs in highly purified T cell populations, i.e., in the absence of phagocytic cells. APCs, on the contrary, diminish Vß8$^+$ T cell loss in culture. When SEB was added to cultures of purified T cells, CsA did not induce deletion (not shown), possibly because this superAg does not crosslink the TcR as effectively as MoAb; however, a mild degree of deletion was observed when APCs were added (Fig. 8).

5. Discussion

CsA, RAPA and FK are drugs that not only suppress immune responses, but that can also alter tolerance to allografts, usually in a beneficial way. The mode of action of these agents on immunological tolerance is not well understood. These agents act on the thymus, CsA and FK causing medullary atrophy (26-28), while RAPA causes cortical atrophy (29). CsA inhibits thymocytic apoptosis in some models (8). However, we and others have also shown effects on peripheral tolerance (reviewed in 30 [Appendix B]). CsA and FK bind to immunophilins (cyclophilin and FK binding proteins [FKBP], respectively) and are thought to act by inhibiting calcineurin, a Ca^{2+}/calmodulin-dependent phosphatase (2-7). The end result is the inhibition of an activation pathway necessary for the production of IL-2 and several other cytokines. RAPA acts by a different mechanism, despite molecular similarity with FK and the ability to bind FKBP. It does not inhibit the initial phase of cytokine production after T cell activation, but it blocks T cell proliferation at the G1-S boundary. RAPA inhibits the activation of p70 S6 kinase (31-33), $p34^{cdc2}$ kinase (34,35), and other enzymes (7,36,37), that play important roles in the control of proliferation of lymphocytes and other cells.

To analyze the effects of these drugs on peripheral T cell deletion, we used three models, superAg immunization, anti-TcR MoAb treatment and rat-into-mouse xenotransplantation. In all these cases, partial deletion of specific

peripheral T cell subsets was detected. There is evidence that superAgs (21), and antibodies against the CD3-TcR complex (38) induce T cell death by apoptosis. Moreover, superAgs induce a state of anergy in residual responding T cells, as previously reported (1 [chapter III],22-24). We found that only CsA was effective at enhancing peripheral T cell deletion. Enhanced deletion of $CD4^+$ T cells was observed after either superAg treatment or after anti-TcR MoAb treatment, but not after xenografting. In addition, combined CsA/anti-TcR-MoAb treatment resulted in severe and prolonged TcR downmodulation in undeleted T cells. In mice that received one injection of MoAb followed by daily CsA injections, T cell numbers did not rise, possibly because CsA inhibits production of $CD4^+CD8^-$ and $CD4^-$ $CD8^+$ T cells in the thymus (10,39); however, the TcR was gradually re-expressed, unless anti-TcR$\alpha\beta$ MoAb was reinjected. In culture, TcR negative cells rapidly re-expressed the TcR. Therefore, the maintenance of maximal T cell deletion requires continuous CsA therapy, while the TcR downmodulation probably depends on the persistence of circulating MoAb. Anti-TcR$\alpha\beta$ MoAb treatment did not induce T cell anergy, irrespective of the presence of CsA. *I.v.* injection of a CD3 MoAb (145-2C-11) had effects similar to those of an anti-TcR$\alpha\beta$ MoAb (H57-579) (our unpublished observations). In vitro, the addition of CsA to cultures of T cells that were stimulated with anti-TcR$\alpha\beta$ MoAb resulted in a marked increase in cell death by apoptosis. Similarly, the combination of KJ16 (anti-Vβ8.1,8.2) and CsA provoked partial deletion of $CD4^+V\beta8^+$ T cells. In

culture, APCs protected T cells from antibody-induced apoptosis. Cell death was most marked in purified T cell populations, and was clearly not the result of phagocytosis of antibody-coated T cells. Instead, APCs protected T cells from apoptosis. Moreover, deletion occurred in circumstances where no proliferation was detected. The effects of SEB in culture were different. When added to purified T cells this superAg did not induce deletion, with or without CsA, possibly because it is not as effective as anti-TcR MoAbs at crosslinking the TcR. A mild degree of deletion was observed when APCs were added to cultures. Therefore, in the case of SEB, the *in vitro* model does not mimic the *in vivo* situation well. The reason for this discrepancy was not elucidated. However, our previous *in vitro* observation on the prevention of anti-TcR MoAbs-induced T cell death by the used of APCs reinforced the hypothesis that deletion is caused by improper T cell activation. Type of APCs involved, as well as the effects of APC irradiation (B cells being highly sensitive), may also be important factors.

The mechanism underlying CsA-enhanced deletion is unknown. Inasmuch as CsA and FK both inhibit calcineurin, it appears that the calcineurin-dependent pathway is not involved. Notably, the effects of CsA on thymocytic and peripheral T cell deletion differ. In the thymus, CsA inhibits anti-CD3 MoAb-induced apoptosis (8), and impairs negative selection in some strains (39,40). However, there appears to be a CsA-resistant deletion pathway in the thymus as well, since CsA delayed but did not abolish clonal deletion in HY-TcR transgenic

mice (9), and failed to prevent endogenous superAg-induced clonal deletion in most mouse strains examined (10,11). Although there is evidence that CsA blocks a calcium-dependent apoptotic pathway involving calcineurin in the thymus (7), we postulate that it enhances apoptosis by an uncharacterized calcineurin-independent pathway in the periphery. This is plausible, since CsA binds to a number of cyclophilins, mostly of unknown function (3,7). Interestingly, for unclear reasons, CsA also enhances anti-CD5 ricin A-chain immunotoxins' effects in human leukemic T cells (41). It should be stressed that the mode of action of CsA is still not fully elucidated. For example, the CsA analog MeAla-6 binds to cyclophilin, does not inhibit calcineurin, and yet does not inhibit CsA action at concentrations where the vast majority of cyclophilin is occupied by the MeAla-6 molecule (7,42).

The mechanisms of peripheral T cell deletion are unresolved. The lack of effect of RAPA suggests that inhibition of proliferation is not a prerequisite factor, and the p70 S6 and $p34^{cdc2}$ kinases probably play no role. This contrasts with a report suggesting that $p34^{cdc2}$ activation is required for apoptosis (43). RAPA, despite a strong anti-proliferative effect, also fails to alter clonal deletion in the thymus (29). Inasmuch as CsA and FK block the secretion of the same family of cytokines (IL-2, IL-3, IL-4, IFNγ and TNFα) (3), it seems unlikely that these mediators are involved. Chlorpromazine, a drug that inhibits SEB-stimulated production of IL-2, IL-4, IFNγ and TNFα, also fails to inhibit SEB-induced

apoptosis (44). Nevertheless, we cannot exclude the possibility that CsA inhibits production of a cytokine (not inhibited by FK) that protects against apoptosis. In any case, SEB-induced clonal expansion and apoptosis appear to be independent of an IL-2 pathway (45). CsA is not the only drug that has been found to enhance peripheral T cell deletion. In particular, glucocorticoids enhance SEB-mediated deletion (46), while the glucocorticoid receptor blocker RU-38486 inhibits this type of cell death, as does the apoptosis inhibitor retinol (44). CsA has a number of complex effects that may influence cell death (reviewed in 3). It inhibits expression of nonlymphokine genes such as *c-myc*, genes of the *src* family, and several other genes that are expressed shortly after T cell activation. Inhibition of *c-myc* is unlikely to explain our results, since this gene can promote apoptosis. CsA may alter the expression of other genes (e.g. *bcl-2* or *fas*) that regulate apoptosis, but to our knowledge this has not been determined. CsA binds to mitochondrial cyclophilins, and inhibits permeability transition. In addition, this drug can alter cell membrane lipids.

The protective role of APCs is of interest. Liu and Janeway (47) found that anti-CD3-TcR-triggered apoptosis in a T_H1 clone was prevented by splenic APCs. In the latter case, IFNγ appeared to play a role in mediating the apoptosis, but this seems unlikely in our model since CsA inhibits secretion of this cytokine. Kabelitz and Wesselberg (48) found that SEA, D, and E (but not SEB) induced apoptosis in a human $CD4^+$ T cell clone in the absence of APCs. The addition of

APCs to cultures resulted in proliferation, but did not prevent apoptosis. They postulate that part of the T cells stimulated by superAgs are programmed to die, while part will proliferate. Possible caveats are that cloned T cells may respond differently than *ex vivo* T cells (49), and there may be species differences in response, especially when we consider the expression of MHC class II molecules by human T cells (MHC class II molecules are not expressed on murine T cells).

We could not demonstrate enhancement of peripheral T cell deletion in response to xenogeneic cell injection. It should be noted that the level of deletion in our xenogeneic transplantation model is low, and the antigen(s) responsible for this process are unknown. Interestingly, limiting dilution analysis suggests that CsA may enhance T cell deletion in some transplantation models (50) and in lymphocytic choriomeningitis virus infected mice (50,51). Therefore, there may be a role for clonal deletion in CsA-promoted transplantation tolerance. In any case, the combination of CsA with anti-TcR or CD3 MoAb therapy should provide a very powerful immunosuppressive combination, in view of the enhanced T cell deletion and TcR downmodulation. The reason for delayed TcR re-expression in CsA/anti-TcR MoAb treated mice is unclear, but this drug was shown to inhibit the expression of TcRα mRNA induced by A23187 in a murine T cell line (3,52). Not surprisingly, we found (unpublished observation) that CsA reduces symptoms related to massive cytokine release, that occurs with therapy with MoAbs that activate T cells such as CD3 (53,54).

We have previously reported on the ability of CsA to inhibit superAg-induced anergy (1 [chapter III]). This effect occurs at lower doses of CsA ($\geq$ 6 mg/kg/d) than enhancement of deletion ($\geq$ 25 mg/kg/d). Our current results add to these observations, and reveal that RAPA and FK have no effect on anergy. It is unclear if the effects of CsA on deletion and anergy are linked, but the lack of activity of FK on anergy again suggests that calcineurin is not involved. This raises the question of whether CsA could sometimes inhibit the development of tolerance. This drug can induce the development of autoimmunity in some models, particularly following syngeneic bone marrow transplantation (reviewed in 30 [Appendix B]), but the mechanisms of action are unclear. RAPA appears to be more effective than CsA at inducing tolerance to allografts (55), and we speculate that this is related to development of T cell anergy, a process not inhibited by RAPA and perhaps promoted by that drug. Chen et al. (56) showed that RAPA-induced allograft tolerance is strain-specific and depends on persistence of alloantigens, a phenomenon observed in thymectomized recipients and consistent with reversible anergy.

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CHAPTER V.

GENERAL DISCUSSION AND CONCLUSIONS

CsA is the most used immunosuppressant today. Its immunosuppressive activity is believed to be caused by the inhibition of interleukin (IL) transcription, principally IL-2 (Elliot, J.F., Lin, Y., Mizel, S.B., *et al.*, 1984; Krönke, M., Leonard, W.J., Depper, J.M., *et al.*, 1984). CsA also showed some intriguing effects on immune tolerance functions (reviewed in Prud'homme, G.J., Parfrey, N.A., and Vanier, L.E. 1991 [Appendix A], and Prud'homme, G.J., and Vanier, L.E., 1993 [Appendix B]). In some experimental conditions, CsA induced permanent allograft tolerance after a short course of CsA treatment. Since the effect of CsA on IL transcription is an active process, its withdrawal should have resulted in prompt rejection of the transplanted organ. CsA could also disrupt immune tolerance and induce syngeneic graft-versus-host disease (GVHD). The mechanisms behind these paradoxical effects of CsA were not well understood. Some studies showed that CsA affected normal thymic functions, thus providing a possible mechanism for the detrimental effects of CsA. However, since the thymus is not the only site where immune tolerance is regulated, CsA probably affected the peripheral tolerance as well. In order to study the effects of CsA on peripheral tolerance mechanisms, adequate models were required. With this objective in mind, we investigated the two forms of peripheral mechanisms recently reported in mice treated with superantigens(superAgs): anergy and peripheral deletion.

SuperAgs stimulate most T cells bearing specific T cell receptor (TcR)-Vβ-chain. *I.v.* injection of superAgs in mice has been shown to induce both anergy and partial peripheral deletion (Rammensee, H.G., Kroschewski, R., and Frangoulis, B., 1989; Rellahan, B.L., Jones, L.A., Kruisbeek, A.M., *et al.*, 1990; Kawabe, Y., and Ochi, A., 1990; Vanier, L.E. and Prud'homme, G.J. 1992). As shown in chapter II, we studied two types of superAgs, Mls-1^a, a surface protein product of the intrinsic mouse mammary tumor virus 7 (MMTV-7) found mainly on B cells, and the staphylococcal enterotoxin B (SEB) produced by *Staphylococcus aureus*. We have determined that once the minimal dose to induce anergy and deletion was attained, further increase of the immunizing dose had no effect. However, we found that multiple injections of SEB caused a more significant deletion than the single dose regimens. This effect was not seen with Mls-1^a.

We observed that anergy was long-lasting (at least 112 days for SEB-immunized mice, and at least 224 days for Mls-1^a-immunized mice). The anergic state could not be reversed by cross-linking of the TcR with matrix bound anti-Vβ-TcR monoclonal antibodies (MoAbs), in the presence of competent antigen-presenting cells (APCs), or by addition of growth factors. The use of thymectomized(Tx)-mice also confirmed the peripheral nature of anergy and deletion in superAgs immunization.

The non-responsive state of anergic T cells had to be distinguished from the

suppression/immunoregulation form of peripheral tolerance. We have found that anergy was not caused by either lack or presence of secreted factors. Based on these studies, it appeared that the superAg models of peripheral tolerance were suitable for the investigation of the effect of CsA on these tolerance mechanisms.

As mentioned above, the thymus of some murine strains treated with CsA failed to delete forbidden T cell clones. This observation lead some researchers to suggest that this defective negative selection was responsible for the syngeneic GVHD. However, our laboratory and others (Prud'homme, G.J., Sander, R., Parfrey, N.A., and Ste-Croix, H, 1991b; Bryson, J.S., Carwood, B.E., and Kaplan, A.M., 1991) have shown that no correlation with the presence of forbidden clones and syngeneic GVHD could be made. Nevertheless, the contribution of a defective thymus in syngeneic GVHD and CsA-induced organ-specific autoimmune diseases has been demonstrated (Sorokin, R., Kimura, H., Schroder, K., Wilson, D.H., and, Wilson, D.B., 1986 and Sakaguchi, S., and Sakaguchi, N., 1988). The discrepancies between the appearance of the forbidden clones and the disease manifestation suggested the involvement of the peripheral tolerance mechanisms. We demonstrated (see chapter III) that low doses of CsA (CsA^{lo}: $\geq$6.25 mg/kg/day *i.p.*) blocked the induction of anergy in superAg-treated mice. This finding provided the first *in vivo* demonstration that CsA blocked anergy. It also lifted some of the questions surrounding the existence of anergy by

showing that the non-deleted fraction of superAg-specific T cells was reactive to the superAg if anergy is prevented.

The enhancement of immune tolerance by CsA was shown in several species (reviewed in Green, C.J., 1988). A short course of CsA treatment induced long-term allograft tolerance. Interestingly, adoptive transfer of $CD4^+$ T cells conferred tolerance to the allograft as well, suggesting a T helper-immunoregulatory mechanism of tolerance (Hall., B.M., Pearce, N.W., Gurley, K.E., and Dorsch, S.E., 1990). Others showed that the deletion of allograft-specific T cells may contribute to the allograft tolerance (Miyagawa, S., Lawen, J.G., Stepkowski, S.M., Kahan, B.D., 1991). Our study of the effect of CsA on the partial peripheral deletion caused by superAg immunization showed that high doses of CsA (CsA^{hi}: $\geq$25 mg/kg/day *i.p.*) markedly enhanced peripheral deletion (chapter III). Under optimal conditions, we found that up to 90% of the superAg-reactive $CD4^+$ T cells could be eliminated.

We repeated the latter experiment with MoAbs directed to the CD3-TcR complex, and found that CsA^{hi} also enhanced the deletion of T cell (chapter IV). In addition, we observed that CsA^{hi} caused a TcR downmodulation in virtually all the remaining T cells. The TcR was generally re-expressed within 14 days.

In order to study the effect of CsA^{hi} enhancement on transplantation antigen, we developed a new model of rat-into-mouse xenotransplant. The injection of WF rat spleen cells into thymectomized B10 mice showed a partial deletion of

$CD4^+/V\beta11^+$ T cells (chapter IV). However, CsA^{hi} treatment had no effect on this model of peripheral deletion.

In vitro studies with matrix-bound MoAbs demonstrated that purified T cells were partially deleted if high concentrations of CsA were used (chapter IV). Interestingly, we found that the deletion could be prevented by the addition of antigen-presenting-cells (APCs). Further analysis of the deleted T cells revealed that they were apoptotic.

We tested the CsA-related immunosuppressants FK-520 (FK) and rapamycin (RAPA) for their effects on anergy and peripheral deletion (chapter IV). We found that both immunosuppressants had no effect on either tolerance mechanisms. Nevertheless, these findings provided some insights to the mechanism(s) involved in CsA modulation of peripheral tolerance. The fact that RAPA inhibits the IL-2 receptor signal, and that FK is a stronger inhibitor of IL synthesis than CsA (including IL-2), imply that CsA-induced apoptosis and anergy inhibition are caused by either the deprivation of an unknown factor, or by an independent molecular mechanism. Since FK-FK-binding-protein complex has a higher affinity for calcineurin than the CsA-cyclophilin complex, it suggests that CsA's effects on anergy and peripheral deletion are conveyed through a novel molecular pathway.

Our understanding of CsA activities on peripheral tolerance mechanisms are crucial for a better management of this drug. The potentiation of peripheral deletion leading to long-term allograft tolerance may revolutionize transplantation medicine. The deletion of all graft-specific T cells prior to transplantation may render the recipient fully tolerant to the allograft, thus abolishing the concept of compatible donor and the need for lifetime treatment with immunosuppressants having long-term toxic side effects. The inhibition of anergy by CsA may be involved in the adverse effects occasionally observed with CsA treatment, such as CsA-induced autoimmune diseases. The improvement of current immunosuppressive therapy can also be explored. Combined treatment with anti-CD3/TcR MoAbs and CsA may significantly enhance the immunosuppression by deleting more T cells than MoAbs alone, and by downmodulating the TcR of the undeleted fraction of T cells. In addition, CsA can reduce the symptoms caused by cytokine release during anti-CD3/TcR MoAbs therapy. Further investigation of the molecular pathway(s) involved in CsA's effects on peripheral tolerance suggested in this study may help the design of new analogs that could selectively potentiate or suppress some of the activities of CsA.

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APPENDIX

APPENDIX A

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Review

CYCLOSPORINE-INDUCED AUTOIMMUNITY AND IMMUNE HYPERREACTIVITY

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1. INTRODUCTION

Cyclosporine (cyclosporin A) is a potent immunosuppressive agent which has been extensively used clinically to treat or prevent allograft rejection, graft-vs-host-disease (GVHD), and autoimmune diseases[1]. In this review, we analyze various experimental and clinical situations where paradoxically cyclosporine (CS) has been shown to either enhance delayed-type hypersensitivity (DTH), enhance alloreactive responses, aggravate autoimmune diseases, or to actually induce specific forms of autoimmunity. The diverse paradoxical effects of this immunosuppressive agent are probably not due to a single mechanism, but rather reflect the ability of CS to cripple various immunoregulatory and tolerance mechanisms. Before discussing the paradoxical effects of cyclosporine, we will briefly review current knowledge of the mode of action of this drug.

2. THE EFFECTS OF CS ON T CELL ACTIVATION, PROLIFERATION AND DIFFERENTIATION

A detailed discussion of the immunopharmacology of CS is beyond the scope of this review, but this topic has been extensively reviewed in several recent monographs[1–8]. Although the pathways leading to T cell activation, proliferation and differentiation are incompletely understood, several key events have been documented (reviewed in Ref. 9). T cell activation is associated with hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), increase in cytoplasmic calcium, and activation of protein kinase C (PKC) and several other kinases (including a CD4/CD8-associated tyrosine kinase). The precise mode of action of cyclosporine is not clear. CS does not interfere with the intracellular rise of Ca^{2+} following activation, but appears to interfere with the subsequent Ca^{2+}-dependent steps[10,11]. CS does not appear to interfere with the hydrolysis of PIP_2 or the activation of PKC[12]. It is well established that CS inhibits IL-2 production by preventing transcription of the IL-2 gene[13]. CS binds to calmodulin[14] and cyclophilin[15] and may inhibit the function of these proteins. Calmodulin regulates enzymes involved in cell activation, while cyclophilin is similar or identical to peptidyl-prolyl-cis-trans isomerase[15]. The latter enzyme, which is inhibited by CS[15], may initiate folding of proteins and expose DNA binding domains. Another possibility is that cyclosporine binds to nuclear receptors which regulate transcription[16,17]. In addition, at very high concentrations CS inhibits RNA polymerases, producing tissue-specific toxic effects[5].

CS inhibits the production by T cells of several cytokines, i.e., IL-2, IL-3, IL-4, IL-5, IFN-gamma, and probably other lymphokines (reviewed in Ref. 8). The effect of CS on IL-2R expression is controversial[7,8,18]. However, IL-2 restores at least partially the proliferative responses of T cells activated in the presence of CS, suggesting that CS inhibits T cell responses primarily by blocking lymphokine synthesis rather than by inhibiting the response to growth factors[8]. CS does not act solely on T cells. There is evidence that CS may interfere with the function of some B cell subsets [20] and antigen presentation by accessory cells[8,21,22]. CS also blocks induced MHC antigen expression under some circumstances. For example,

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Halloran *et al.*[23] found that CS prevents the hyperexpression of MHC antigens in some tissues which is induced by the administration of LPS (which stimulates IFN-gamma production).

(a) T cell responses resistant to CS

As mentioned above, T cells activated in the presence of CS are still at least partially capable of expressing IL-2R and of proliferating in the presence of IL-2. This view is corroborated by the findings of Granelli-Piperno[24] who recently demonstrated by *in situ* hybridization that CS has no effect on IL-2 receptor mRNA expression *in vivo*. Thus, the T cells of CS-treated subjects may be activated by antigen and poised to respond as soon as CS is withdrawn. Moreover, there is evidence that T cell priming *in vivo* to alloantigens can be mediated by an IL-2-independent CS resistant pathway. Pereira *et al.*[25] found that the T cells of CS-treated mice which had been injected with allogeneic cells appeared to have undergone priming and differentiation. Upon culturing *in vitro* in the presence of CS, they found that the cells from CS-treated animals manifested a vigorous proliferative response that could not be inhibited by the addition of a large panel of anti-cytokine monoclonal antibodies. Thus, this type of T cell growth is IL-2-independent, but the stimulating growth factor(s) is/are unknown. Similarly, Chisholm and his colleagues demonstrated that CS failed to prevent many of the early signs of T cell activation in GVHD, and cardiac allograft rejection[26,27]. In humans, it has been found that the activation of T cells through the CD28 pathway (with monoclonal antibody 9.3) is resistant to CS[28].

Although CS inhibits the generation of CTLs in a primary MLC[1], this may be secondary to the inhibition of IL-2 production. CS does not appear to prevent the maturation of pre-CTLs to CTLs[29]; however, there are conflicting data on this subject. Studies by Hess[7] provided evidence on the ability of CS to prevent development of IL-2 responsiveness of CTLs in a human MLC system. However, the inhibitory effect of CS was dose dependent, such that CTL responses could be restored by adding IL-2 to cultures when CS concentrations were relatively low. Hegg *et al.*[30] reported that the maturation of CTLs in the presence of CS required the presence of both IL-2 and IL-4. In any case, the inhibitory effects of CS on CTLs *in vitro* are often not apparent *in vivo*. For example, Noble and Steinmuller[31] found that CS blocks IL-2 production but not the tissue destruction induced by cytotoxic T cells. Moreover, CS appears to have little effect on the priming of T cells mediating protective immunity to viral antigens[32].

There are several reports suggesting that CS does not interfere with the function of T suppressor cells (reviewed in Refs. 1 and 33). The presence of CS in primary MLC leads to the expression of antigen non-specific suppressor cells. In rat allograft recipients, CS therapy has been reported to permit long term tolerance and to induce antigen-specific suppressor cells. However, CS appears to inhibit suppressor cells in some autoimmune diseases. The effects of CS on suppressor cells are obviously complex and will be discussed below.

(b) Immune responses enhanced by CS

Studies on the mechanism of action of CS indicate that this drug acts very early in the process of T cell activation. As pointed out by Borel[1], CS has to be administered during the sensitization period and for a significant length of time in order to exert its inhibitory effects. When CS is administered late or for only a short period of time, immune responses are often only mildly suppressed, unaffected, or enhanced[1,8]. The enhancement of immune responses by CS has been reported in numerous situations (see below). It should be noted that similar forms of enhancement may occur with other immunosuppressants as well, but a discussion of other drugs is beyond the scope of this review.

3. AUGMENTATION OF DTH RESPONSES WITH CS

In most situations CS suppresses DTH responses; however, Thomson and Aldridge[34] showed that a 5-day course of CS given from the time of immunization of guinea pigs with ovalbumin resulted in a significant augmentation of DTH reactions to this antigen elicited 14 days later. Augmentation of tuberculin-like hypersensitivity in guinea pigs[35] and DTH responses to sheep RBC[36,37] in mice have also been reported. Several factors influence the outcome of immunization including the species immunized, the temporal relationship between CS and antigen administration, the type of antigen, and the dose of CS. The mechanism of DTH enhancement is unclear, but it has been proposed that CS augments immune responses by inhibiting the function of suppressor cells[38].

Recent studies with Th1 and Th2 T cell clones (see Ref. 39 for review) raise interesting questions on the enhancing effect of CS on some DTH responses. Th1 clones, that appear to mediate DTH responses (at least in mice), secrete IFN-gamma which inhibits the proliferative response of Th2 cells to either IL-2 or IL-4[40,41]. Conversely, Th2 cells secrete a factor that inhibits lymphokine production by Th1 cells[42]. In addition, Th2-derived IL-4 antagonizes some of the effects of IFN-gamma[43]. Thus, Th1 and Th2 cells are antagonistic, i.e., they behave as "suppressor" cells in

a limited and selective way. *In vivo*, this Th1/Th2 antagonism may apply since it has been observed that immunization with an antigen in a way that tends to promote an antibody response is often associated with unresponsiveness in a DTH response to the same antigen (i.e. immune deviation or split-tolerance) and vice versa[44]. CS markedly enhances DTH responses to *Leishmania major* in BALB/c mice[45] (these mice usually produce high IgE levels, but poor DTH responses to *L. major* antigens[46]), and CS enhances DTH to SRBC in mice[37], while concurrently antibody production to *L. major* and SRBC are profoundly suppressed respectively. Thus, CS can induce a shift towards a more Th1 dependent response. However, CS sometimes promotes IgE production [47] (which is Th2 dependent); and *in vitro*, Th2 clones appear to be more resistant than Th1 clones to the action of CS[48]. Thus, although CS appears capable of altering Th1/Th2 antagonism, it is unclear why Th1-dependent responses (DTH) are sometimes favored.

4. ENHANCEMENT OF GVHD AND ALLOGRAFT REJECTION IN CS-TREATED SUBJECTS

The capacity of CS to prevent allograft rejection and allogeneic GVHD has been extensively documented [1,5,8]. There are specific instances, however, where CS treatment appears to have augmented alloreactive responses.

GVHD in particular may be associated with or follow CS treatment. In rats, local GVHD (induced by injecting parental T cells into F1 recipients) can be greatly augmented by administering a single dose of CS at the same time as cell transfer[1,49]. Systemic disease in the parent-into-F1 GVHD model is suppressed by CS; however, withdrawal of the drug results in an accelerated lethal GVHD[26]. In humans, bone marrow transplant (BMT) recipients treated with CS for a year or more frequently develop an acute form of GVHD shortly after CS is withdrawn[50]. This late onset of an acute form of GVHD is not often seen in patients treated with methotrexate. Moreover, a GVHD-like condition in human recipients of solid organ allografts has been related to tapering doses of CS[51,52], suggesting that autoaggressive T cells were generated during the period of CS treatment. Interestingly, CS inhibited the development of tolerance to allografts in baboons treated with total lymphoid irradiation (TLI)[53], and in dogs which received allografts after autologous BMT[54].

It is noteworthy that lymphocytic infiltration is frequently seen in surviving allografts of CS-treated animals and patients, and renal allografts are often rejected despite CS treatment[1,8]. These findings are consistent with the view that although CS blocks T cell effector function, it does not inhibit the earliest stages of T cell activation, as discussed previously. Furthermore, alloreactive T cells may undergo clonal expansion in CS-treated recipients, through the IL-2-independent, CS-resistant pathway of T cell activation/differentiation[25]. The combination of TLI or BMT with CS may compound the problem by stimulating the production of new T cells from the thymus which fail to be tolerized in the presence of CS (see below).

5. AUTOIMMUNE DISEASES AGGRAVATED BY CS

CS has proven effective in the treatment of several autoimmune diseases. In experimental animals or humans, CS suppresses the expression of adjuvant arthritis, experimental allergic encephalomyelitis (EAE), autoimmune uveitis, spontaneous type I diabetes (insulin-dependent), rheumatoid arthritis, primary biliary cirrhosis, and several other autoimmune diseases (reviewed in Refs. 1 and 55). Nevertheless, in several cases CS has been shown to aggravate autoimmunity, and we will discuss some of the autoimmune diseases in this group.

Kaibara *et al.*[56] demonstrated that collagen-induced arthritis in rats is suppressed when CS treatment begins at the time of immunization with type II collagen, but disease is enhanced when CS is administered after immunization, i.e., in the preclinical or clinical phase of the disease. Enhancement of arthritis was accompanied by enhanced DTH response to type II collagen, while antibody responses were either suppressed or unaffected. This form of enhanced DTH with suppressed antibody production suggests that collagen-induced arthritis is another model where CS can alter Th1/Th2 antagonism.

CS can suppress a primary episode of EAE; however, CS has been shown to alter the subsequent course of EAE (or related autoimmune diseases) in rats and guinea pigs[58–63]. In rats, EAE can occur as a single episode followed by resistance to disease or as a chronic relapsing disease (CREAE), depending on the immunization protocol. In a model of EAE in Lewis rats which usually occurs as a single episode, Polman *et al.*[58] found that low doses of CS did not prevent the initial attack of EAE and induced a relapsing form of EAE. Relapse was associated with severe CNS lesions. In a different rat model which is usually characterized by CREAE, Feurer *et al.*[59] found that treatment with CS frequently resulted in hyperacute attacks, instead of a relapsing course. This may have resulted from alterations of the balance between effector and suppressor populations[60]. Reiber and his

colleagues[61,62] demonstrated that acute EAE in guinea pigs could be suppressed by CS, whereas the same dose of CS given in mature animals with relapsing remitting disease resulted in clinical deterioration. Interestingly, guinea pigs injected with the tolerogenic MBP-derived peptide S42 do not develop EAE, and are resistant to subsequent attempts to induce this disease[63]. However, a single dose of CS on day 15 after S42 immunization prevents the development of resistance to EAE induction[63]. Despite these interesting findings the effects of CS on suppressor cells in EAE remain obscure. This is highlighted by the fact that Ellerman *et al.*[64] generated T suppressor cell lines from the draining lymph nodes of rats immunized with MBP by culturing cells in a medium supplemented with CS. The T suppressor cells generated by this method (that are obviously CS resistant) have a $CD4^+$ phenotype and can inhibit the passive transfer of EAE.

CS may enhance some forms of autoimmune thyroiditis. For example, when CS is administered to obese strain (OS) chicken embryos, there is subsequently a more severe thyroiditis and higher titers of antithyroglobulin antibodies[65]. In humans, CS therapy may aggravate Grave's disease[66].

In low dose streptozotocin-induced diabetes, a disease thought to have an autoimmune component, CS treatment causes a dose-dependent enhancement of beta-cell destruction and hyperglycemia[67–70]. However, the interpretation of these results is clouded by the probable toxicity of CS for beta islet cells.

SDA (Smyth delayed amelanotic) chickens are prone to develop posterior uveitis, which appears to have an autoimmune component. This ocular pathology is decreased by administering CS from the time of hatching; however, termination of CS therapy results in enhanced uveitis (compared to controls) 4 to 8 weeks later[71,72]. Thus, although CS prevents acute disease, autoaggressive T cells are probably generated which give rise to a "rebound" phenomenon as soon as CS is withdrawn. Nevertheless, CS has proven highly effective in the treatment of autoimmune uveitis in humans.

Coxackie B3-induced myocarditis in mice is thought to have an autoimmune component[73–75]. This autoimmune component is mediated by $CD8^+$ T cells and is resistant to CS therapy[74]. In fact, Estrin *et al.*[74] found that CS therapy can increase mortality 2–4 times in this disease model. This increased mortality cannot be attributed directly to enhanced viral infection, since mice injected with both CS and rabbit antithymocyte serum were protected from myocarditis. Estrin and Huber[73] identified two CTL populations in coxsackie virus-infected mice. A $CD8^+$ T subset reacts only with uninfected heart cells and is clearly autoaggressive, while a $CD4^+$ cytolytic T subset reacts only with infected cells. Both types of T cells can contribute to myocarditis, but the $CD8^+$ cells appear to cause most of the tissue injury. Interestingly, CS inhibits the production of the virus-specific T cells, but not of the autoaggressive $CD8^+$ cells. The CS-resistant cytolytic T cells identified in this model appear similar to those recently described by Pereira *et al.*[25] in a transplantation model.

The mechanisms by which CS augments these autoimmune diseases are unclear. It is noteworthy that DTH responses are important in EAE and probably in several other autoimmune diseases. Thus, CS may enhance autoimmunity for the same reasons that it enhances some DTH responses. Since T suppressor cells have been described in several autoimmune diseases, and appear to confer disease resistance[57,64], it is plausible that CS inhibits the function of these cells. However, numerous reports indicate that Ts cells are often resistant to CS[1,33,76,77], and as mentioned previously CS has been used *in vitro* to generate Ts cell lines. Presumably, there exists CS-sensitive and CS-resistant suppressor cells (of either T or non-T lineage), which are of variable importance depending on the disease model. The role of suppressor cells in CS-induced autoimmunity is discussed in another section of this review (below). Alternatively, CS could aggravate disease by promoting the activation of T cells through a CS-resistant pathway, or by preventing the development of tolerance to antigens (see below).

6. CYCLOSPORINE-INDUCED AUTOIMMUNITY (CSIA)

In all the previous examples CS augmented an induced immune response or a spontaneous autoimmune response; however, there are at least three experimental models of primary cyclosporine-induced autoimmune disease: syngeneic GVHD (SGVHD) which has now been reported in rats, mice and humans; a systemic autoimmune disease in irradiated CBA/N mice; and organ specific autoimmune diseases in mice.

(a) CS-induced SGVHD

Glazier *et al.*[78] found that rats which are lethally-irradiated, reconstituted with syngeneic bone marrow, and treated with CS for at least 3 weeks develop a lethal GVHD-like syndrome after CS is withdrawn. This finding has been confirmed by other groups[79–81], and we have been able to induce this disease in several rat strains[81]. In mice, CS-induced syngeneic GVHD was initially reported by Cheney and Sprent[83], a finding which could not be reproduced by some investigators[84]. However, recent studies by us[85] and Bryson *et al.*[86] reveal that only some mouse strains are susceptible, e.g. DBA/2, and the disease only occurs in young mice. A similar disease occurs in humans

following withdrawal of CS treatment in recipients of autologous or syngeneic bone marrow[87,88]. It should be stressed that SGVHD has also been reported in the absence of CS-therapy in both humans[89] and experimental animals[90]. However, the occurrence of SGVHD is unpredictable in subjects that have not received CS, and the disease may be mild and transient. Thus, CS treatment provides a reproducible method of inducing a severe form of SGVHD.

A contributing role for viruses in CS-induced SGVHD cannot be ruled out, but the disease has been observed in apparently virus-free rats. CS-induced SGVHD cannot be attributed to genetic differences between donors and recipients, or to alterations of bone marrow cells during the process of transplantation, since we (unpublished) and others[78,79] have observed the same disease in sublethally irradiated rats treated with CS. Several features support an autoimmune etiology, including a thymic dependency[79,82,91], the adoptive transfer of disease with T cells or thymocytes[79,91,92], and the identification of cytolytic Ia-specific T cells in rats[93] and humans[87].

(b) Tissue lesions and MHC expression in CS-induced SGVHD

The lesions documented by Glazier *et al.*[78] and others[81,94] reveal that CS-induced SGVHD is virtually identical to allogeneic GVHD. Rats show obvious signs of GVHD which include erythematous ears, hair loss, weight loss, and diarrhea. Most rats die 2–4 weeks after cessation of CS treatment. Histologically, there is involvement of the skin, tongue, liver and GI tract. Lymphocytes infiltrate target tissues and are often seen in close proximity to apoptotic epithelial cells.

The thymus plays a key role and must be in the field of irradiation for disease to occur[82]. CS-treated syngeneic BMT recipients show a marked thymic medullary involution with loss of medullary Ia^+ cells[81,82]. Interestingly, although we find that CS treatment causes an almost complete loss of Ia^+ thymic medullary cells in rats[81], this is not the case in mice where we find that MHC class II antigens continue to be expressed at a high level[81]. We also find that the sequence of lesions and alterations of MHC antigen expression (except in the thymus) are similar in allogeneic GVHD and SGVHD[81,95].Thus, lymphoproliferation (in secondary lymphoid tissues) with numerous blasts cells constitutes the earliest lesions. This is followed shortly by MHC class I hyperexpression and MHC class II induction on epithelial cells, as well as increased numbers of MHC class II positive macrophage/dendritic cells in several sites. Tissue lesions (in both allogeneic and CS-induced syngeneic GVHD) follow MHC class II induction on epithelial cells. Surprisingly, class II positive dendritic cells in the heart and other non-epithelial tissues do not appear to elicit a response, suggesting that the target antigens may be epithelial-cell-derived peptides presented by class II MHC molecules. This hypothesis is consistent with the findings of Gaspari and Katz[96] who reported that although Ia^+ keratinocytes do not stimulate primary alloreactive T cell responses, they can nevertheless act as targets for class II-restricted CTLs. This view is also consistent with the identification of class II-restricted CTLs in rats and humans with CS-induced SGVHD[87,93]. In acute allogeneic GVHD the relative importance of class I and class II disparities is controversial, but we have been able to induce this disease across class II disparities, and to prevent the disease with anti-Ia antibodies[97]. Thus, similar effector cells may mediate acute allogeneic and acute syngeneic GVHD.

(c) Chronic CS-induced SGVHD

Beschorner *et al.*[94] have recently reported transition from acute-type SGVHD to a chronic-type SGVHD following withdrawal of CS. It has been suggested that longer courses of CS treatment are more likely to give a chronic type of SGVHD[98]. Beschorner *et al.*[94] found that chronic SGVHD was characterized by fibrosis of the dermis and lamina propria in the skin and tongue, submucosal fibrosis in the small intestine, and salivary gland infiltrates with fibrosis and loss of ducts and glands. These investigators[94] suggest that immediately post-CS, SGVHD is primarily acute with epithelial infiltrates of $CD8^+CD4^-$ T cells, and lamina propria infiltrates that include $CD4^+CD8^+$ T cells consistent with immature thymocytes. There is rapid transition to chronic-type SGVHD with a residual mucosal infiltrate dominated by $CD4^+CD8^+$ T cells, while the lamina propria infiltrate is now dominated by $CD4^+CD8^-$ T cells. It should be noted that we have not observed this chronic form of SGVHD in the rats we have studied, but this is probably due to the fact that we have employed a short course of CS treatment (3 weeks) in our studies[81,85]. The reported predominance of $CD8^+$ T cells in the lesions of acute SGVHD is difficult to reconcile with our observation of a correlation between MHC Class II antigen induction and the pattern of tissue lesions. However, in rats with CS-induced SGVHD, Hess *et al.*[93] identified $CD8^+$ CTLs with anti-Ia specificity. These cytolytic cells were unusual in that Ia-specificity is usually associated with a $CD4^+$ phenotype, and the cells reacted with Ia antigens on target cells of several MHC haplotypes.

(d) Adoptive transfer of CS-induced SGVHD

Adoptive transfer studies have only partially clarified the role of $CD4^+$ and $CD8^+$ T cells in this disease. Sorokin *et al.*[79] were able to adoptively transfer

SGVHD with relatively small numbers of $CD4^+$ cells (depleted of $CD8^+$ cells and B cells), while $CD8^+$ T cells were much less effective. In contrast, Fischer *et al.*[92] demonstrated a requirement for both $CD4^+$ and $CD8^+$ cells to transfer SGVHD effectively. However, small numbers of contaminating $CD4^+$ or $CD8^+$ T cells could expand rapidly *in vivo* and obscure the interpretation of these adoptive transfer experiments. Moreover, the role of $CD4^+CD8^+$ cells has not been addressed in these studies.

(e) CS-induced autoimmunity in CBA/N mice

There have been only a few reports of CS-induced SGVHD in mice[83,85,86], presumably due to the difficulty of inducing disease in this species. Marcos and his colleagues (reviewed in Ref. 99) described a variant of CS-induced SGVHD in CBA/N mice. This disease was only observed in CBA/N mice, which carry the *xid* mutation, and differed in several respects from the SGVHD observed in rats. CBA/N mice received 800 cGy of total body irradiation with lead shielding of one leg, followed by i.p. injection of CS at a dose of 15 mg/kg/day from the day of irradiation. Surviving mice developed a systemic disease characterized by weight loss, dull fur, and an asymmetrical form of polyarthritis that evolved to chronic joint deformities. The mice also developed proteinuria and had immunoglobulin deposits in the glomerular mesangium and basement membrane. Irradiated CS-treated CBA/N mice had severe thymic atrophy with decreased relative numbers of $CD4^+CD8^+$ and increased numbers of bright $V\beta8^+$ ($F23.1^+$) T cells. These CS-induced thymic changes are clearly different from those reported by others in syngeneic bone marrow chimeras (to be described later). Irradiated CS-treated CBA/N mice had evidence of polyclonal B-cell hyperactivity, antinuclear autoantibodies, and other autoantibodies resembling the disease seen in lupus-prone mice. Thus, it appears that in irradiated CBA/N mice, CS caused the development of a chronic GVHD-like disease associated with a lupus-like disease. This is surprising since the *xid* mutation protects mice that spontaneously develop lupus. Interestingly, lupus-like disease has been reported in mice with chronic allogeneic GVHD that did not receive CS[100]. The latter lupus-like disease appears to result from the activation of B cells by Ia-specific alloreactive T cells.

(f) Effects of CS on thymocytic and peripheral T cell populations

We examined the effects of CS on thymocytic maturation in rats and mice, and on clonal deletion in mice[85]. We found, in agreement with other reports[101,102] that CS causes a partial arrest of thymocyte maturation at the stage of $CD4^+CD8^+$ cells, such that fewer mature-type (single positive) T cells are seen in the thymus.

In the blood and spleen of CS-treated syngeneic BMT recipients we observed an increase in the numbers of $CD4^+CD8^+$ T cells[85]. Approximately 19% of the double-positive peripheral T cells of rats also expressed a thymocyte marker ($OX7^+$), suggesting that they may represent incompletely matured thymocytes. Other investigators have also reported increased numbers of $CD4^+CD8^+$ peripheral T cells in CS-treated rats[103,104]. Moreover, Beschorner *et al.*[94] have identified $CD4^+CD8^+$ T cells infiltrating the mucosa of the tongue of rats with CS-induced SGVHD, as described previously, suggesting that double-positive T cells play an important role in the pathogenesis of this disease. The possibility that the peripheral double-positive cells we have identified represent mature activated T cells must also be considered, since double positive T cells have been reported following the activation of $CD4^+$ T cells, but these activated cells are not $OX7^+$ (unpublished observation).

We were able to induce SGVHD in several rat strains; however, of 7 mouse strains we tested only DBA/2 mice developed disease. Our results are similar to those of Bryson *et al.*[86] who were able to induce disease in DBA/2 mice but not in BALB/c mice. The reason for these differences in disease susceptibility are not clear, since we found that CS induced similar changes in thymic and peripheral T cell populations in all the mouse strains tested. However, studies in rats and mice reveal that apparently minor alterations in the protocol for administrating CS have a major impact on disease expression. For example, a delay of 3 days between BMT and the administration of CS was sufficient to prevent disease expression[86], and only young rats and mice appear susceptible to SGVHD[82,86].

(g) Effects of CS on clonal deletion in syngeneic bone marrow chimeras (SBMC)

We examined the expression of TCR Vβ elements in 3 strains of mice where clonal deletion has been observed, i.e., C57BR mice, CBA/J mice, and DBA/2 mice[85]. Although CS had marked effects on thymocyte maturation, it did not alter the TCR-mediated events which result in the clonal deletion of $V\beta17a^+$, $V\beta8.1^+$ and $V\beta6^+$ T cells. DBA/2 mice with evidence of SGVHD had very low numbers of $V\beta6^+$ T cells consistent with normal clonal deletion. Interestingly, Gao *et al.*[102] reported that CS interferes with the deletion of $V\beta11^+$ T cells in $I\text{-}E^+$ mice. According to hybridoma studies, $V\beta11^+$ T cells appear to have a relatively low affinity for I-E, and *in vivo* these cells are not normally completely deleted[102]. We speculate that CS may alter deletion of clones with a relatively low affinity for antigen, but has no effect on deletion of

clones with a high affinity for self antigens. We were inhibited from performing similar studies in rats due to the absence of adequate markers. Thus, the question of clonal deletion in the rat model of SGVHD is unresolved.

Contrary to our findings, Jenkins *et al.*[101] reported the presence of Vβ17a$^+$ T cells in the J11d$^-$ thymocyte subpopulation (mostly single-positive cells) of SBMC C57BR mice treated with CS. These investigators did not examine peripheral T cells. The reason for this discrepancy with our results is unclear. However, if intrathymic clonal deletion fails then undeleted clones should be apparent in extrathymic sites. We did not observe increased numbers of undeleted Vβ17$^+$ T cells in the splenic T cells of CS-treated C57BR mice. We speculate that the Vβ17$^+$ T cells identified by these investigators were not fully mature and were still capable of undergoing clonal deletion. (perhaps extrathymic deletion).

The recent finding that CS interferes with apoptosis in the thymus[105] appears at variance with our observation of clonal deletion in CS-treated mice. Although it is generally assumed that self-antigen specific thymocytes are eliminated by apoptosis, this is not the only possible mechanism. Notably, Nakashima *et al.*[106] observed that the incubation of mouse thymocytes with a cloned thymic epithelial cell line (TEL-2) resulted in the elimination of CD4$^+$CD8$^+$ thymocytes by a process apparently different from apoptosis, i.e., live CD4$^+$CD8$^+$ thymocytes were integrated in the cytoplasm of TEL-2 cells where they gradually degenerated. The importance of apoptosis has been questioned[107], and Quackenbush and Shields[108] have recently reported *in vivo* labelling studies which do not support the occurrence of a high rate of apoptosis in the thymus. In any case, clonal deletion is clearly only one mechanism responsible for tolerance, and clonal anergy or suppressor cells[109–111] may be equally important. CS appears to inhibit the development of both T cell clonal anergy and T suppressor cells (see below).

(h) CS-induced organ-specific autoimmunity

An altogether different CS-induced autoimmune syndrome has been described in mice by Sakaguchi and Sakaguchi[112,113]. When CS was administered to newborn mice for a few days after birth, the mice subsequently developed organ-specific autoimmune diseases such as gastritis, oophoritis, thyroiditis, insulitis, and other lesions. These autoimmune diseases were associated with the presence of organ-specific autoantibodies. They found that CS caused loss of CD4$^-$CD8$^+$ and CD4$^+$CD8$^-$ thymocytes and peripheral lymphoid atrophy. Autoimmunity also resulted when nude mice were transplanted with the thymus of CS-treated donor mice[113]. Autoimmune disease was prevented when CS-treated newborn mice were inoculated with splenic T cells from normal syngeneic mice. Thymectomy immediately following withdrawal of CS produced a higher incidence of autoimmune disease, as well as disease in a wider spectrum of organs. Interestingly, a similar form of autoimmunity occurs in rats or mice thymectomized in the neonatal period (e.g. day 3 thymectomy), suggesting that early thymic failure leads to immunoregulatory defects. Recently, it has been demonstrated by Smith *et al.*[114] that the neonatal thymus is "leaky", i.e., it does not support normal clonal deletion as seen in adults. Thus, murine neonates have peripheral undeleted T cells with a potential for autoreactivity. Early thymectomy appears to freeze the T cell repertoire, such that there are unusually high numbers of undeleted T cell clones in peripheral T cell populations[114]. Whether or not these undeleted clones cause autoimmunity has not been directly demonstrated. It is interesting that these undeleted clones do not appear to cause disease in normal mice, suggesting that they undergo clonal anergy or are inactivated by suppressor cells.

7. EFFECTS OF CS ON IMMUNE REGULATION AND TOLERANCE

The ability of suppressor cells to prevent CS-induced autoimmunity is suggested by some studies. Passive transfer of disease is only achieved in recipients that have been irradiated or treated with cyclophosphamide[115]. More direct evidence of suppression comes from the studies of Sorokin *et al.*[79] and Fischer *et al.*[115] who demonstrated that the injection of syngeneic normal-rat T cells into CS-treated syngeneic BMT recipients prevents disease. Fischer *et al.*[115] found that normal-rat CD4$^+$ T cells had to be cotransferred with normal-rat CD8$^+$ T cells in a ratio that approximates the ratio in unfractionated splenocytes in order to affect inhibition of the transfer of SGVHD. The regulatory CD4$^+$ T cells were found to be OX22$^-$. Moreover, Fischer and Hess[116] recently reported that marrow from rats of 6 months of age was virtually incapable of eliciting SGVHD after BMT and CS therapy. This resistance was due to the presence of mature T cells in the marrow of animals 6 months of age. Although the marrow of younger animals also contained T cells, the regulatory activity was absent.

There are many similarities between suppression in SGVHD and in several autoimmune diseases. Cohen and his colleagues[57] have described innate or induced resistance to autoimmune disease in EAE, thyroiditis, and adjuvant arthritis. In EAE, CD4$^+$[64] and CD8$^+$[117] suppressor cells have been cloned. These cells appear to suppress by reacting against the clonotypic determi-

nants of autoaggressive T cells. This view is supported by the ability to induce specific resistance in animals to an autoimmune disease with "T cell vaccination", i.e., by injecting healthy recipients with an inactivated autoaggressive T cell clone or by injecting numbers of these T cells which are too small to transfer disease[57]. It is noteworthy that CS can induce a chronic relapsing form of EAE in Lewis rats, suggesting that CS may prevent the generation of suppressor cells. $CD4^+$ suppressor cells also regulate expression of autoimmune diabetes in NOD mice[118], but the effect of CS on these cells is unknown.

CS has been found to alter tolerance in several situations. CS interferes with the development of tolerance in mice injected with high doses of xenogeneic RBCs[36,37]; which is a model where the importance of suppressor T cells has been amply demonstrated. CS has also been reported to interfere with the development of neonatal tolerance to allogeneic cells[119], but in this case the relative contributions of clonal deletion, clonal anergy, and suppressor cells are not clear. CS prevents the development of specific forms of tolerance to allografts[53,54]. Durable and specific tolerance to allografts can be achieved with TLI in baboons; but Myburgh[53] found that CS inhibited the development of this type of tolerance. Supralethal total body irradiation and reconstitution of the host with autologous bone marrow produces a limited period where tolerance to allograft is easily induced[54]. However, Bachavaroff *et al.*[54] found that CS prevented the development of this form of tolerance to renal allografts in dogs. We have obtained similar results in mice (see below). These results differ from situations where CS has been shown to induce tolerance to allografts[33]; however, tolerance has usually been induced in recipients that did not receive TLI or BMT.

CS may abrogate some forms of tolerance by preventing the development of clonal anergy. Jenkins *et al.*[109] found that *in vitro* CS inhibited T cell clonal anergy, which was induced by incubating T cells with chemically modified APCs and antigen. We recently developed an *in vivo* model of clonal anergy by grafting allogeneic skin to mice which had been lethally irradiated and reconstituted with T-depleted syngeneic bone marrow. In control mice (no CS) skin graft survival time was increased and MLC responsiveness was reduced, without evidence of clonal deletion. However, a 14-day course of CS following skin grafting abrogated this tolerance (unpublished observation). We also found that CS inhibits the development of T cell clonal anergy in Mls-1^b mice injected with Mls-1^a cells (unpublished observation).

CS could also affect the development of tolerance by inhibiting the production of T cell-derived IL-2 and IFN-gamma, since these two lymphokines stimulate natural suppressor (NS) cell function. We have reported on the contribution of NS cells in local GVHD (parent-into-F1)[120]; and the latter reaction can be enhanced by CS[49].

8. SUMMARY AND CONCLUSIONS

Cyclosporine (CS) is a potent immunosuppressive agent which under some circumstances paradoxically augments DTH responses, aggravates some autoimmune diseases, and induces specific forms of autoimmunity. The enhancement of DTH and other immune responses is closely related to the timing of CS administration relative to immunization. CS inhibits IL-2 production (and several other lymphokines) at a pretranscriptional level, but does not usually prevent the antigen-specific priming of T cells, such that T cells may be poised to respond as soon as CS is withdrawn. Thus, accelerated GVHD and allograft rejection may occur after withdrawal of CS. CS has been shown to aggravate and/or induce relapse in several autoimmune diseases including collagen-induced arthritis, EAE, autoimmune thyroiditis, uveitis in SDA chickens, and an autoimmune form of myocarditis in mice. CS may enhance immune responses by inactivating suppressor cells, by altering Th1/Th2 antagonism (e.g., CS promotes a protective Th1-type response in BALB/c mice infected with *Leishmania major*), or by promoting T cell activation through a CS-resistant IL-2-independent T cell activation/differentiation pathway.

At least three forms of CS-induced autoimmunity have been described: Syngeneic or autologous GVHD which occurs in CS-treated syngeneic or autologous bone marrow transplant recipients after CS is withdrawn in rats, mice, and humans; a systemic autoimmune disease with polyarthritis and glomerulonephritis which occurs in irradiated CBA/N mice treated with CS; and organ-specific autoimmune diseases which occur in mice treated with CS during the neonatal period. The precise mechanisms by which CS induces these autoimmune diseases are not clear; however, CS affects immune tolerance at three levels. CS induces thymic medullary involution with loss of medullary Ia^+ cells, and appears to at least partially block the transition from double positive ($CD4^+CD8^+$) to single positive (mature type) thymocytes. In syngeneic bone marrow chimeras, CS appears to inhibit the intrathymic deletion of clones with relatively low affinity, but not those with high affinity, to self antigens. CS appears to inhibit the action of suppressor T cells which normally maintain an innate form of resistance to autoimmunity. Finally, CS has been shown to prevent the development of T cell clonal anergy. There is redundancy in immune tolerance mechanisms, i.e., clonal deletion, clonal anergy, and suppressor cells can each maintain tolerance to

similar antigens, such that it is likely that CS must cripple more than one tolerance mechanism for autoimmunity to occur.

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APPENDIX B

CLINICAL IMMUNOLOGY AND IMMUNOPATHOLOGY
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SHORT ANALYTICAL REVIEW

Cyclosporine, Tolerance, and Autoimmunity

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INTRODUCTION

Cyclosporine A (CsA) is a potent immunosuppressive agent that is increasingly used in the treatment of autoimmune diseases, but which can sometimes paradoxically enhance immune responses and induce specific forms of autoimmunity (1). For example, CsA can induce syngeneic or autologous graft-vs-host disease (GVHD) in radiation bone marrow chimeras in rats (2–6), mice (7–9), and humans (10, 11). To induce GVHD-like disease, CsA must usually be administered for 3 or more weeks starting soon after bone marrow transplantation, and disease occurs after CsA is withdrawn. This is clearly an autoimmune disease, which can be adoptively transferred with T-cells. CsA can induce a systemic autoimmune-like disease in irradiated CBA/N (*xid*-bearing) mice (12), and provokes organ-specific autoimmunity in mice treated with CsA during the neonatal period (13, 14). In some circumstances, CsA can aggravate collagen-induced arthritis in rats, experimental allergic encephalomyelitis (EAE) in rats and guinea pigs, autoimmune thyroiditis in OS chickens, uveitis in SDA chickens, and virus-initiated autoimmune myocarditis in mice (reviewed in Ref. 1). In contrast, CsA can induce long-term tolerance to allografts in rats and other species (15). Thus, CsA can either block or enhance immune responses and/or tolerance depending on the experimental model.

TOLERANCE AND INTRATHYMIC CLONAL DELETION

The mechanisms by which CsA can alter immunologic tolerance are not clear, but it appears that this drug affects tolerance induction in both the thymus and the periphery. In rats, CsA causes severe thymic medullary involution (5, 16), and particularly a loss if Ia^+ medullary dendritic cells. There is a partial arrest in the maturation of thymocytes at the $CD4^+CD8^+$ stage (8). In CsA-treated rats, $CD4^+CD8^+$ peripheral T-cells resembling thymocytes are observed in the periphery (8, 17–19), suggesting that immature thymocytes are emigrating from the thymus. Moreover, in rat syngeneic radiation bone marrow chimeras treated with CsA, Ia-reactive autoaggressive cytotoxic T lymphocytes have been identified (20). Similar cells have also been identified in humans (10). In mice, CsA has been reported to induce thymic changes similar to those reported in rats (7, 21), but we found much milder thymic (medullary) alterations in mice than those in rats (5, 8). It has been proposed that CsA-induced thymic abnormalities are associated with a failure of clonal deletion. In mice, CsA blocks the apoptosis of T-cells induced by CD3 antibodies (22). However, it is not clear that this represents a relevant model for negative selection in the thymus, since in bcl-2 transgenic mice anti-CD3-induced apoptosis is prevented, but negative selection still occurs (23). Jenkins *et al.* (21) found undeleted mature-type (single-positive) thymocytes, i.e., $V_\beta 17a^+$ cells, in CsA-treated C57BR ($I\text{-}E^+$) syngeneic mouse bone marrow chimeras (8). Gao *et al.* (24) report small numbers of undeleted peripheral T-cell receptor (TCR)-$V_\beta 11^+$ T cells in I-E-bearing CsA-treated syngeneic mouse bone marrow chimeras. However, we did not find elevated numbers of T-cells bearing forbidden TCR's in the spleen of either CsA-treated CBA/J, C57BR, or DBA/2 syngeneic bone marrow chimeras (8). Recently, Bryson *et al.* (25) reported that CsA blocks clonal deletion in some mouse strains but not in others. Interestingly, of several mouse strains we treated with CsA, only DBA/2 mice developed syngeneic GVHD. Since clonal deletion was observed by us (8) and others (25) in DBA/2 mice with CsA-induced syngeneic GVHD, it appears that a failure of intrathymic clonal deletion is not the key event in this disease in mice, but clonal deletion has not been studied in rats. The interpretation of results on thymic clonal deletion is complicated by our recent observation that CsA can enhance T-cell deletion in the periphery, as described below.

Whatever the mechanisms, it is clear that thymic effects are important in cyclosporine-induced autoimmunity. In rats, CsA-induced syngeneic GVHD does not occur in thymectomized recipients (3) and only occurs when the thymus is in the field of irradiation (16). Beschorner and his colleagues (16) showed that CsA

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treatment (with or without mediastinal irradiation) causes loss of thymic medullary epithelium, Hassall's corpuscles, and MHC Class II antigen expression. Recovery is rapid after withdrawal of CsA, except in rats that have received thymic irradiation (16), a feature that may be important in the pathogenesis of CsA-induced syngeneic GVHD. Moreover, Beschorner *et al.* (26) found that if CsA-treatment in nonirradiated rats is followed by thymectomy, the rats frequently develop a syngeneic GVHD-like disease. No disease is seen in sham-thymectomized rats. They propose that the peripheral effector cells in the post-CsA thymectomy group were derived from the CsA-altered thymus, and that the absence of syngeneic GVHD in their sham-thymectomized group is due to the production of regulatory cells by the regenerating thymus (26). In mice, Sakaguchi and Sakaguchi (14) showed that engrafting of the thymus from CsA-treated mice, whether newborn or adult, into syngeneic adult athymic nude mice could produce organ-specific autoimmune diseases. In another study (13), these authors reported that treatment of mice with CsA during the first seven days of life (Days 0–6) resulted in organ-specific autoimmunity, and that the frequency, variety, and severity of lesions was markedly accentuated by Day 7 thymectomy. These authors proposed that CsA acts on the thymus by preventing the development of suppressor cells, since autoimmune disease was prevented when CsA-treated newborn mice were inoculated with splenic T-cells from normal syngeneic mice (13).

CsA ALTERS DEVELOPMENT OF PERIPHERAL T-CELL DELETION AND ANERGY

Recently we found that CsA alters the development of peripheral T-cell tolerance (27). In our experiments we used two superantigens, staphylococcal-enterotoxin B (SEB) and Mls-1^a (a product of the intrinsic mouse mammary tumor virus 7), which stimulate TCR-V$\beta 8^+$ and CD4^+/TCR-V$\beta 6^+$ T cells, respectively. These superantigens are known to cause both a partial elimination and a long-term *in vitro* anergy in their targeted T-cell subpopulations (28–32). We found that CsA markedly enhances T-cell deletion in superantigen-immunized mice, such that deletion was frequently three- to fourfold greater. For example, following a single iv injection of 10^7 Mls-1^a cells (from donor DBA/2 mice, H-2^d Mls-1^a) into CsA-treated recipient BALB/c mice (H-2^d, Mls-1^b) we frequently observed less than 1% CD4^+/$V_\beta 6^+$ T-cells, and T-cells expressing non-Mls-1^a-reactive V_β elements were not deleted. Similarly, deletion of $V_\beta 8^+$ T-cells, particularly the CD4^+ subset, was markedly enhanced by CsA treatment in SEB-immunized mice (27). Usually, immunization with superantigens results in a proliferative phase in reactive T-cells, followed by partial deletion (29–31). CsA abolished the proliferative phase, but enhanced deletion, suggesting that these two events are not necessarily linked.

The degree of peripheral T-cell deletion was similar in thymectomized and nonthymectomized mice. These results establish that CsA can enhance peripheral T-cell clonal deletion. In contrast, in CsA-treated superantigen-immunized mice the remaining (undeleted) superantigen-reactive T-cells failed to develop anergy (*in vitro* proliferative unresponsiveness), unlike the T-cells of non-CsA-treated mice (27). The degree of tolerance in CsA superantigen-treated mice appears to depend on a balance between these opposing effects on deletion and anergy. In fact, under conditions where T-cell deletion was not extensive, the T-cell responses to superantigens were usually much higher in CsA-treated vs non-CsA-treated superantigen-immunized mice. Higher doses of CsA were required to enhance T-cell deletion (25 mg/kg/day ip or more), than to impair anergy induction (6 mg/kg/day or more) (27). Consequently, proliferative responses to superantigens were enhanced with low-dose CsA treatment (lack of anergy), but were depressed with high-dose drug treatment (extensive deletion). Rapamycin, a potent immunosuppressive agent that acts by a different mechanisms, had no effect on either clonal deletion or anergy in superantigen-immunized mice (our unpublished observation). Whether CsA can induce similar effects in response to conventional antigens, rather than superantigens, is unknown but clearly of great interest. The possibility that a defect in anergy could result in autoimmunity is discussed in a section below.

HYPOTHESES TO EXPLAIN THE EFFECTS OF CsA ON PERIPHERAL T-CELL ANERGY AND DELETION

The mechanisms(s) by which CsA can prevent the induction of peripheral T-cell anergy is not clear. CsA is known to block a Ca^{2+}-dependent signal. However, the mechanisms of anergy induction in superantigen-immunized mice are poorly understood, and it is not clear how CsA acts in this process. New insights into the mechanism of action of CsA may help to clarify this question in the near future. CsA forms a complex with cyclophilin, and this complex can then bind to and block the activity of calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase (reviewed in 34). It is postulated (34) that calcineurin plays a role in activating a cytoplasmic component of NF-AT. This factor then migrates into the nucleus and binds with another component to form the active nuclear NF-AT factor. The NF-AT site and other sites of the IL-2 gene enhancer are sensitive to the action of CsA. However, the role of nuclear factors in the induction of anergy is still unknown. CsA may inhibit the production or activity of DNA-binding proteins that are critical in inducing the anergic state, but this is clearly speculative.

The work of Shi *et al.* (22) demonstrates that CsA blocks anti-CD3-induced programmed cell death (apoptosis) in the thymus. However, CsA does not prevent radiation-induced apoptosis in mature T-cells (35).

Nevertheless, it was surprising to find that CsA could enhance peripheral T-cell deletion. One possibility is that peripheral T-cells are not eliminated by apoptosis, but the recent studies of Kawabe and Ochi (31) suggest that SEB induces peripheral T-cell apoptosis. Interestingly, we found that the stimulation of T-cells *in vitro* with matrix-bound anti-TCRαβ antibodies in the presence of CsA induces extensive T-cell death after 48–72 hr in culture, apparently by apoptosis. Over the same time period, cell death was minimal in unstimulated T-cells, while, in the absence of CsA, anti-TCRαβ antibodies stimulated strong proliferation. Rapamycin prevented T-cell proliferation, but did not cause T-cell death under similar conditions (our unpublished observations). These preliminary data suggest that CsA may enhance apoptosis in mature antigen-reactive T-cells.

We can only speculate on the mechanism by which CsA enhances peripheral T-cell deletion, but Duke and Cohen (36) have shown that IL-2 deprivation of activated T-cells results in death by apoptosis. Since CsA blocks IL-2 production and interferes with anergy induction, the T-cells activated by superantigens may be left with no source of IL-2. This could possibly result in apopotosis and loss of reactive T-cells in CsA/super Ag-treated mice.

CsA HAS PARADOXICAL AND POORLY UNDERSTOOD EFFECTS ON SUPPRESSOR CELLS

In addition to effects on clonal deletion and anergy, CsA may also act on suppressor T-cells or promote suppressor activity by acting on other cells (reviewed in Ref. 37). In several autoimmune diseases CsA appears to inhibit suppressor cell function and to provoke or aggravate disease (1, 37). On the other hand, $CD4^+$ suppressor T-cells appear to maintain the state of tolerance observed in CsA-treated rats that are recipients of allogeneic heart transplant (15). Suppressor T-cells, comprising both $CD4^+$ and $CD8^+$ cells, may also prevent the expression of CsA-induced syngeneic GVHD (see below). In these rat models, the existence of suppressor or regulatory T-cells is strongly supported by the adoptive transfer of tolerance with these cells. However, the current poor understanding of the phenotypic and functional characteristics of suppressor T-cells makes it difficult to interpret these results. Moreover, T-helper-1 (Th1) clones and T-helper-2 (Th2) clones are mutually suppressive and may be responsible for some of these suppressive phenomena, as discussed in the next section. The existence of suppressor cells does not exclude other tolerance mechanisms, and in fact, a recent study suggests that deletion may play a role in CsA-induced tolerance to allografts (38). Ito *et al.* (38) found that the injection of KCl-extracted donor antigen (HAg) and CsA resulted in donor-specific tolerance to heart allografts in rats. Limiting dilution analysis revealed a marked decrease in the frequency of cytotoxic T-cell reactive to donor-type target cells in CsA-treated recipients. In addition, in tolerant rats both $CD4^+$ and $CD8^+$ T-cell subsets specifically suppressed the proliferative response of recipient T-cells against donor-type stimulator cells. Thus, CsA administration was characterized by either clonal deletion or inactivation of effector cells, as well as increased suppressor cell activity.

ALTERATION OF Th1/Th2 ANTAGONISM AND ENHANCEMENT OF DELAYED-TYPE HYPERSENSITIVITY (DTH) RESPONSES

It is now well established that Th1 and Th2 clones have antagonistic effects (39). Th1 cells produce IL-2 and interferon -γ and mediate DTH responses; while Th2 cells produce IL-4, IL-5, IL-6, and IL-10 and stimulate antibody production. Th1-derived interferon -γ can inhibit Th2 activity, while Th2-derived IL-10 can prevent Th1 activation by acting indirectly on macrophages. In this way, Th1 and Th2 cells can act as suppressor cells, at least in a limited way. CsA may alter the balance between Th1 and Th2 responses. For example, CsA markedly enhances delayed-type hypersensitivity (DTH) responses to *Leishmania major* in BALB/c mice (40) [these mice usually produce high IgE levels, but poor DTH responses to *L. major* antigens (41)]; and CsA enhances DTH responses to sheep RBC in mice (42), while concurrently antibody production to *L. major* and sheep RBC are profoundly suppressed respectively. Thus, CsA can induce a shift towards a more Th1-dependent response (DTH). However, CsA sometimes promotes IgE production (43) (which is Th2 dependent); and *in vitro,* Th2 clones appear to be more resistant to the action of CsA than Th1 clones (44). Because of these contradictory observations, it is unclear why Th1 responses (DTH) are sometimes favored. Recently, Bretscher and Hasele (45) reported that CsA can facilitate the induction of DTH when antigen is administered in a way that normally stimulates antibody production, but inhibits DTH when mice are immunized in a way that normally promotes DTH. They suggest that DTH is favored by a "weaker" antigenic stimulation, while antibody production requires a larger number of responding T-cells or higher doses of antigen. Thus, CsA may act by reducing the number of T-cells that are initially stimulated by an antigen, i.e., a situation which mimics a weaker immunogenic stimulus.

A shift in Th1/Th2 balance could be detrimental in some autoimmune diseases. For example, Kaibara *et al.* (46) demonstrated that collagen-induced arthritis in rats is suppressed when CsA treatment begins at the time of immunization with type II collagen, but disease is enhanced when CsA is administered after immunization, i.e., in the preclinical or clinical phase of the disease. Enhancement of arthritis was accompanied by enhanced DTH response to type II collagen, while an-

TABLE 1
Autoimmune Diseases Induced or Aggravated by CsA

Experimental model or clinical disease	Comments	References
Syngeneic or autologous GVHD	Occurs after withdrawal of CsA treatment in rats and mice; similar disease reported in humans	1–11, 16, 17, 20, 25, 52, 53
Organ-specific autoimmune diseases in mice (thyroiditis, gastritis, orchitis, etc.)	Occurs after neonatal treatment of mice with CsA (Days 0–6); aggravated by Day 7 thymectomy	13, 14
Systemic autoimmune disease (polyarthritis, glomerulonephritis)	CsA treatment of irradiated CBA/N (*xid*-bearing) mice	12
Collagen-induced arthritis in rats	Disease is aggravated when CsA is administered immediately in the preclinical phase of arthritis; DTH to type II collagen is increased	46
EAE	CsA induced hyperacute attacks with death in rats	47
	Low doses did not prevent EAE and induced relapsing disease with severe CNS lesions in rats	60
Thyroiditis	When administered to OS chicken embryos there is subsequently a more severe thyroiditis	61
	CsA can aggravate Graves' disease in humans	62
Myocarditis in mice	CsA aggravates coxsackie B3-initiated autoimmune myocarditis in mice; CsA-resistant $CD8^+$ T-cells kill uninfected heart cells	63, 65
Uveitis in SDA chickens	CsA aggravates the autoimmune uveitis which spontaneously develops in these chickens (however, CsA is very effective in treating human uveitis)	66, 67
Streptozotocin-induced diabetes in mice	CsA causes a dose-dependent enhancement of beta-cell destruction and hyperglycemia (a caveat is that CsA is toxic to islet cells)	68, 69

tibody responses were either suppressed or unaffected. This form of enhanced DTH with suppressed antibody production suggests that collagen-induced arthritis is a model where CsA alters Th1/Th2 antagonism to the advantage of Th1 cells. DTH responses to myelin basis protein are thought to be important in EAE, and it is noteworthy that in a rat model of chronic relapsing EAE Feurer *et al.* (47) found that treatment with CsA frequently resulted in hyperacute attacks with death, instead of a relapsing course. Several other organ-specific autoimmune diseases can also be aggravated by CsA (1), and it is not unlikely that alterations in Th1/Th2 antagonism play a role in these situations.

TOLERANCE VS AUTOIMMUNITY

CsA/antigen-induced elimination of peripheral T-cells and/or generation of suppressor cells may represent mechanisms of tolerance to allografts. On the other hand, as mentioned above, CsA treatment can induce autoimmunity. Cyclosporine-induced syngeneic (or autologous) GVHD, in particular, has been extensively studied. The pathogenesis of this disease remains to be elucidated, but we postulate that a blockage of anergy induction may be important. In CsA-induced syngeneic GVHD, the effector T-cells may consist of T-cells that are normally tolerized primarily by anergy rather than intrathymic deletion. This hypothesis is consistent with the observation that in some mouse strains CsA treatment induces this disease, despite apparently normal intrathymic clonal deletion (25). There is increasing evidence that anergy is an important mechanism of tolerance, particularly for antigens that are not expressed in the thymus (reviewed in Refs. 48, 49). Anergy may serve as a backup tolerance mechanism for T-cells that escape clonal deletion (50, 51). The importance of anergy has been most clearly demonstrated in transgenic mice, where in some cases unresponsiveness is secondary to a functional inactivation of T-cells (48, 49, 51). Although a failure of anergy induction could be one mechanism by which CsA can provoke the generation of autoaggressive T-cells, this does not explain the observation that adoptive transfer of syngeneic GVHD can be prevented by coinjection of normal syngeneic T-cells (3). In fact, Fisher *et al.* (52) found that in normal rats populations of T-cells downregulate the activity of autoaggressive T-cells and prevent the development of syngeneic GVHD. These regulatory T-cells are present in the secondary lymphoid organs of normal rats (52), in the bone marrow of old rats (6 month), but not in the bone marrow of young rats (4 week) (53), and include cells of both the $CD4^+$ and $CD8^+$ phenotype (52, 53). Therefore, CsA may exert two effects that are both required to cause autoimmunity, i.e., blockage of anergy and loss of suppressor T-cell function. Alternatively, instead of preventing anergy, CsA could be blocking the intrathymic deletion of autoreactive T-cell clones. We

TABLE 2
Abrogation of Tolerance and Paradoxical Effects Induced by CsA

Experimental model (disease relevance)	Effects of CsA	References
Syngeneic radiation bone marrow chimeras (syngeneic GVHD)	Thymic medullary atrophy and loss of medullary Ia^+ cells	5, 7, 16
	Partial arrest of thymocytic differentiation at the $CD4^+CD8^+$ stage	4, 8, 21, 24
	CsA inhibits clonal deletion in some mouse strains but not others; lack of correlation with disease expression	8, 21, 24, 25
	Loss of $CD4^+$ and $CD8^+$ suppressor cells	3, 52, 53
	Peripheral autoreactive cytotoxic T cells	10, 20
Treatment of mice with CsA during the neonatal period (organ-specific autoimmune diseases)	Depletion of single-positive $CD4^+$ and $CD8^+$ thymocytes and peripheral T cells	13
	Transplant of thymus of CsA treated mouse (newborn or adult) into nude mouse results in autoimmunity	14
	Loss of suppressor cells in the thymus and periphery of CsA-treated mice	13, 14
CD3 mAb induced cortical thymocytic apoptosis in mice (? model of clonal deletion)	Inhibits apoptosis	22
Anergy in Th1 $CD4^+$ T-cell clones induced by presentation of antigen by fixed cells	Blocks anergy induction	33
Superantigen-induced anergy of peripheral T-cells in mice (? loss of anergy leads to autoimmunity)	Blocks anergy induction	27
Alteration of Th1/Th2 antagonism (possible relevance to collagen-induced arthritis and/or other organ-specific diseases)	Increased DTH response, and decreased humoral response, to *Leishmania major* in BALB/c mice	40
	Increased DTH response, and decreased humoral response to sheep RBC in mice	42
	Aggravates collagen-induced arthritis and increases DTH to type II collagen in mice	46
	Facilitates DTH when antigen is administered in a way that normally stimulates antibody production	45
Neonatal tolerance to alloantigens	Inhibits tolerance induction	54
Tolerance to renal allografts in baboons treated with total lymphoid irradiation	Prevents induction of tolerance	57
Tolerance to renal allografts in dogs that have been lethally irradiated and reconstituted with autologous bone marrow	Prevents induction of tolerance	58
Injection of adult mice with allogeneic cells	Generation of alloreactive responder cells through an IL-2-independent pathway	59

were unable to identify increased numbers of forbidden clones in our experiments, but only superantigen-reactive T-cells were enumerated. We cannot exclude a defect in the deletion of other clones, perhaps those with a lower affinity/avidity for self antigens. Recent studies demonstrate that there is redundancy in immune tolerance mechanisms, such that clonal deletion, clonal anergy, and suppressor cells may all be capable of maintaining tolerance to similar antigens. Thus, it seems likely that CsA has to cripple more than one tolerance mechanism for autoimmunity to occur.

It is noteworthy that mice treated with CsA during the neonatal period often develop organ-specific autoimmune diseases in adult life (13, 14). Similarly, CsA prevents the development of neonatal tolerance to alloantigens (54), but the mechanisms involved in this type of tolerance are complex and incompletely understood. Clonal anergy and intrathymic clonal deletion have both been shown to play a role (55, 56), and CsA could interfere with either of these processes. Moreover, in neonatally treated mice, CsA may prevent the generation of suppressor cells (13, 14). In some circumstances, CsA can also abolish the induction of tolerance to alloantigens in adult mice, e.g., in CBA mice injected iv with A/J bone marrow cells (54). CsA has been shown to inhibit the tolerance to allografts which normally occurs after total lymphoid irradiation in baboons (57) or after lethal irradiation and autologous bone marrow transplantation in dogs (58). In all these situations, the mechanism of action of CsA remains to be clearly established.

Another aspect to consider is that some immune responses are resistant to CsA (reviewed in Ref. 1). For example, Pereira *et al.* (59) found that the T-cells of CsA-treated mice which had been injected with allogeneic cells appeared to have undergone priming and differentiation. Upon culturing *in vitro* in the presence of CsA, they found that the T-cells from CsA-treated animals manifested a vigorous proliferative response that could not be inhibited by the addition of antibodies

against IL-2 and several other cytokines. Thus, CsA-resistant T-cells were generated that appeared to respond by an IL-2-independent pathway. Activation through the CD28 pathway is also known to be CsA resistant. These results suggest that the administration of CsA leads to the generation of IL-2-independent effector T-cells by alternative pathways that could play a role in graft rejection and autoimmune diseases.

SUMMARY AND CONCLUSIONS

A large number of studies have documented the effects of CsA on immunologic tolerance. Initially, these studies concentrated on the ability of CsA to induce tolerance to allografts, particularly in rats. However, it has become apparent that CsA can sometimes block the induction of tolerance, and provoke or aggravate specific autoimmune diseases in several species (Table 1).

CsA can inhibit at least three processes thought to contribute to tolerance (Table 2). In the thymus, CsA has been reported by some investigators to block negative selection, but surprisingly, large numbers of undeleted or "forbidden" T-cells are rarely observed in the periphery. In mature T-cells (extrathymic), CsA can block the induction of anergy which occurs after immunization with superantigens, and *in vitro* this drug can block anergy induction in Th1 clones which occurs when antigenic peptides are presented by metabolically inactive (treated with a fixative) antigen-presenting cells. However, CsA can also enhance the deletion of peripheral superantigen-reactive T-cells, when the drug is administered at high doses. Thus, responses to superantigens can be either enhanced or inhibited depending on the protocol of CsA administration. Whether or not these phenomena apply to conventional antigens has not been determined. Numerous studies demonstrate that CsA treatment can either enhance or depress suppressor T-cell function in various experimental models. The interpretation of data on suppressor cells is complicated by the current poor understanding of the function of the cells. In CsA-induced syngeneic (or autologous) GVHD, CsA may inhibit both T-cell anergy induction and the generation and/or function of suppressor cells. CsA can alter Th1/Th2 antagonism, such that DTH responses are enhanced. Enhanced Th1 activity could explain the deterioration that CsA sometimes provokes in autoimmune diseases where DTH is important, e.g., collagen-induced arthritis and EAE. CsA can prevent the development of neonatal tolerance. The latter effect may account, at least in part, for the induction of organ-specific autoimmunity observed after treating mice with CsA during the neonatal period. However, in all the situations mentioned the effects of CsA on tolerance cannot be easily explained by a single mechanism. There is redundancy in the immune tolerance mechanism, such that clonal deletion, clonal anergy, and suppressor cells may all be capable of maintaining tolerance to similar antigens. Thus, it is likely that CsA must inhibit more than one of these mechanisms for autoimmunity to occur.

The outcome of treatment with CsA on tolerance is influenced by several factors including the dose, the timing of administration, and the type of antigenic stimulation. The effects of CsA on immunologic tolerance are likely to be of importance over and beyond the direct immunosuppressive effect, and could in some cases determine the longterm outcome of therapy with this drug.

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